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Award Number: W81XWH-07-1-0061

TITLE: The Function of PTP1B in Neuroendocrine Differentiation of Prostate Cancer

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REPORT DATE: January 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE 1 Jan 2009		2. REPORT TYPE Annual		3. DATES COVERED 1 Jan 2008 – 31 Dec 2008	
4. TITLE AND SUBTITLE  The Function of PTP1B in Neuroendocrine Differentiation of Prostate Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-07-1-0061	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)  Jiaoti Huang, M.D., Ph.D.  E-Mail: <a href="mailto:JiaotiHuang@mednet.ucla.edu">JiaotiHuang@mednet.ucla.edu</a>				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)  The University of Rochester Rochester, NY 14627				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT  Purpose: The goal of the project is to identify the molecular mechanisms responsible for therapeutic failure in prostate cancer patients receiving hormonal therapy Scope: The scope of the project is to use prostate cancer cell lines in in-vitro cell culture systems to study the complex signal transduction pathways that may be responsible for the neuroendocrine differentiation of prostate cancer cells, particularly the relationship of PTP1B to IL-8 signaling through its receptors CXCR1 and CXCR2, to IGF-1 receptor signaling through PI3 kinase/AKT/mTOR pathway and to androgen receptor signaling. Major findings: 1. We have shown that IGF-1 receptor may be a critical player in androgen-withdrawal-induced neuroendocrine differentiation of prostate cancer cell line LNCaP. 2. We have shown that IGF-1 receptor is activated by the expression of a protein tyrosine phosphatase PTP1B in LNCaP cells, which may be responsible for the activation of AKT signaling pathway. In addition, our results suggest that this activity of PTP1B appears to be independent of its tyrosine phosphatase enzymatic activity.					
15. SUBJECT TERMS Prostate cancer, hormonal therapy, neuroendocrine cells, PTP1B, IGF-1 receptor, androgen receptor, cancer stem cells					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	37	19b. TELEPHONE NUMBER (include area code)

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## **INTRODUCTION:**

The ultimate goal of our research is to study the molecular mechanisms responsible for therapeutic failure in prostate cancer (PC). PC, when localized, can be treated with surgery or radiation. Once the tumor is advanced or metastatic, hormonal therapy, consisting of androgen ablation and/or anti-androgen, is the treatment of choice. Unfortunately, although nearly all patients respond to this treatment initially, the tumor invariably recurs and enters the androgen-independent (hormone-refractory) stage, for which there is no effective therapy. We hypothesize that the reason for the eventual failure of the therapy is because PCs contain neuroendocrine (NE) cells in addition to the more abundant secretory type cancer cells. Unlike the secretory type cancer cells that express androgen receptor (AR) and depend on androgen for proliferation, NE cells are negative for AR and are androgen-independent, as we have demonstrated in a recent publication (1). We hypothesize that hormonal therapy induces differentiation of some secretory type cancer cells to NE cells and these NE cells survive hormonal therapy and are responsible for tumor recurrence. We further hypothesize that hormonal therapy causes changes in multiple signaling pathways in PC cells, and coordination and cross-talk of the different pathways lead to NE differentiation. The main objective is to study the mechanism of action of PTP1B in androgen withdrawal-induced NE differentiation, in relation to other important intracellular signaling pathways. Three tasks were proposed in the original proposal:

- 1). The function of PTP1B in the regulation of IL-8 signaling in PC;
- 2). The function of PTP1B in IGF-1 receptor signaling through PI3K/AKT/mTOR pathway;
- 3). The function of PTP1B in androgen receptor signaling.

## **BODY:**

### **1. Research accomplishments associated with task 1: The function of PTP1B in the regulation of IL-8 signaling in PC**

Task 1 has been completed and reported in last year's annual report

### **2. Research accomplishments associated with task 2: The function of PTP1B in IGF-1 receptor signaling through PI3K/AKT/mTOR pathway**

When the proposal was being submitted, we had obtained preliminary data showing that androgen withdrawal in LNCaP cells induces activation of the PI3 kinase-AKT-mTOR pathway, which is required for NE differentiation induced by androgen withdrawal. Since PI3K-AKT-mTOR pathway is classically activated by a cell surface protein tyrosine kinase receptor, we hypothesize that androgen withdrawal activates such a receptor, leading to activation of the PI3K-AKT-mTOR pathway, resulting in NE differentiation.

A leading candidate protein tyrosine kinase receptor is IGF-1 receptor, which has been implicated in many aspects of PC. We have shown that IGF-1 can induce PI3K-

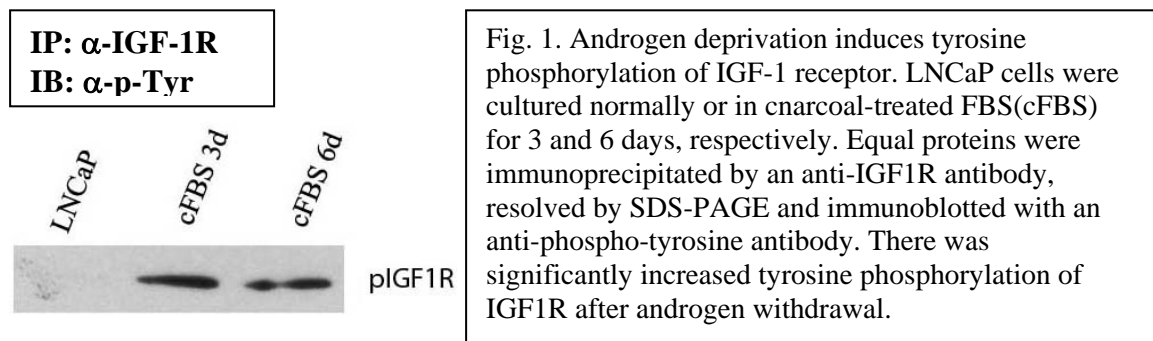
AKT-mTOR pathway and NE differentiation in LNCaP cells. A manuscript describing these important findings was published in the Journal of Biological Chemistry (2).

The results associated with this task are summarized as follows:

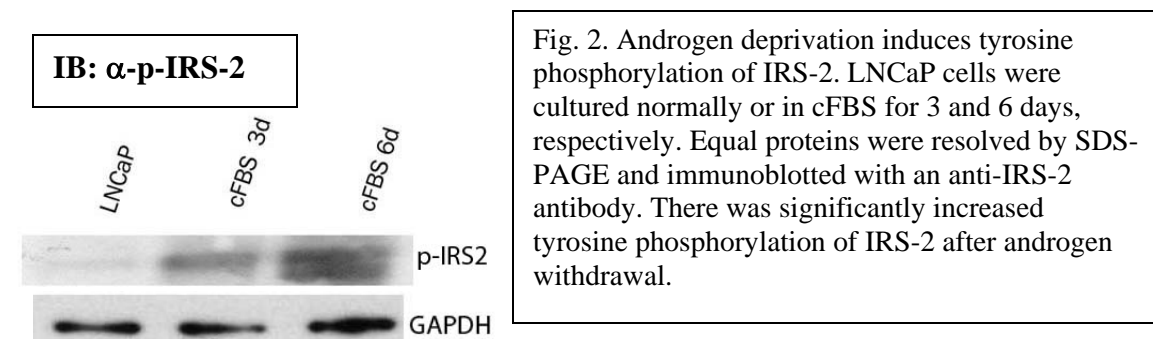
**Task 2a: Confirming that IGF-1R is activated by androgen withdrawal of LNCaP cells.** To accomplish this task, we proposed to induce neuroendocrine differentiation in LNCaP cells by androgen withdrawal and study if IGF-1 receptor and IRS-2 are activated and whether their activation is required for neuroendocrine differentiation

#### **Accomplishments:**

We cultured LNCaP cells in androgen-deprived media (charcoal-treated FBS) for 3 and 6 days, respectively. Equal proteins from each sample were immunoprecipitated by anti-IGF1R and anti-IRS-2 antibodies, respectively and separated by SDS-PAGE. Proteins are transferred to nitrocellulose membranes and immunoblotted with an anti-phospho-tyrosine antibody (p-Tyr-100). As demonstrated in Figure 1, under conditions that induce neuroendocrine differentiation of LNCaP cells (cFBS), tyrosine phosphorylation of IGF-1 receptor is significantly increased, indicating activation of this tyrosine kinase receptor.



Consistent with the above finding, we have also found that IRS-2, a major downstream molecule of IGF-1 receptor, is also tyrosine phosphorylated (Figure 2) after androgen withdrawal, indicating that the signaling pathway of IGF-1 receptor is activated under conditions of neuroendocrine differentiation.



The above results confirmed our hypothesis that androgen withdrawal activates IGF-1 receptor and its signaling pathway. To determine if activation of IGF-1R pathway is required for androgen withdrawal-induced NE differentiation, we first used an anti-

IGF-1 receptor antibody. We showed that this antibody appeared to inhibit tyrosine phosphorylation of IGF-1 receptor in androgen-deprived media at 3 days (Figure 3). However, the addition of the anti-IGF-1R antibody did not show a significant effect at 6 days after androgen deprivation, possibly due to degradation of the antibody (Fig. 3)

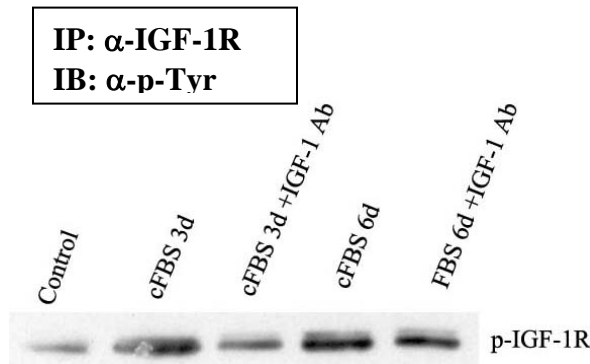


Fig. 3. The effects of an-anti-IGF-1R antibody on tyrosine phosphorylation of IGF-1R after androgen deprivation. LNCaP cells were cultured normally (control) or in charcoal-treated FBS (cFBS). Androgen deprivation for 3 and 6 days increases tyrosine phosphorylation of IGF-1R. The effect at 3 days is neutralized by an anti-IGF-1R antibody but the antibody's effect is less obvious at 6 days.

We further tested if the anti-IGF1 receptor antibody may inhibit the signaling pathway induced by the activation of the IGF-1 receptor after androgen deprivation. We studied the phosphorylation of AKT, a major signaling molecule downstream of IGF-1 receptor, as a surrogate of the signaling strength of IGF-1 receptor. Our study shows that androgen withdrawal induces the phosphorylation of AKT at 3 and 6 days, as we have reported in a previous publication (2). However, we found that the anti-IGF-1 receptor antibody did not decrease the phosphorylation of AKT. In fact, AKT phosphorylation after 3 days of androgen withdrawal appears to increase in the presence of the anti-IGF-1 receptor antibody (Fig. 4).

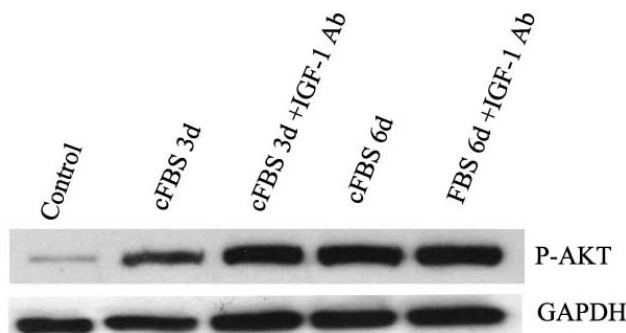


Fig. 4. Anti-IGF-1R antibody does not inhibit phosphorylation of AKT after androgen deprivation. LNCaP cells were cultured normally (control) or in charcoal-treated FBS (cFBS). Androgen deprivation for 3 and 6 days increases phosphorylation of AKT. Anti-IGF-1 receptor antibody does not decrease AKT phosphorylation.

This finding suggests two possibilities. One is that AKT phosphorylation is independent of IGF-1 receptor activation. However, we favor the alternative interpretation that the anti-IGF1R antibody does not block the signaling pathway induced by IGF-1R under the experimental conditions. This is more possible as our experiment lasts for several days and the antibody, even if possessing receptor blocking function, may have been degraded in the tissue culture system.

We therefore tried to use a chemical inhibitor of IGF-1 receptor, cyclolignan PPP, to determine if inhibition of IGF-1R activation may decrease AKT phosphorylation. In the first experiment, we showed that cyclolignan PPP inhibits the tyrosine phosphorylation of IGF-1R by approximately 50% (Fig. 5).

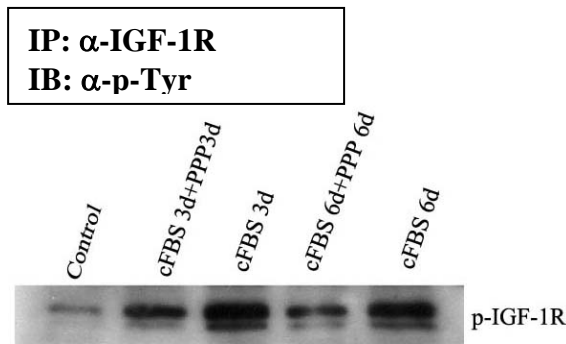


Fig. 5. Cyclolignan PPP inhibits the activation of IGF-1 receptor after androgen deprivation. LNCaP cells were cultured normally (control) or in charcoal-treated FBS (cFBS). Androgen deprivation for 3 and 6 days increases tyrosine phosphorylation of IGF-1R, which is inhibited by IGF-1R inhibitor cyclolignan PPP

We next studied if cyclolignan PPP can inhibit the activation of AKT. As shown before, androgen deprivation for 3 and 6 days significantly increases AKT phosphorylation. However, in the presence of cyclolignan PPP, AKT phosphorylation is inhibited (Fig. 6), indicating that increased phosphorylation and activation of AKT results from activation of IGF-1R.

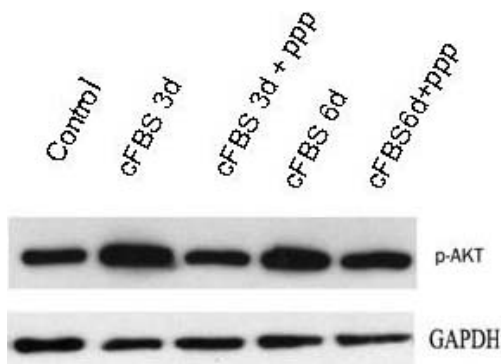


Fig. 6. Cyclolignan PPP inhibits the activation of AKT after androgen deprivation. LNCaP cells were cultured normally (control) or in charcoal-treated FBS (cFBS) in the absence or presence of Cyclolignan PPP. Equal amounts of proteins were resolved by SDS-PAGE and immunoblotted with an anti-pAKT antibody. Androgen deprivation for 3 and 6 days increases tyrosine phosphorylation of IGF-1R, which is inhibited by IGF-1R inhibitor cyclolignan PPP

In our previous publication (2), we have shown that androgen withdrawal in LNCaP cells leads to increased phosphorylation of S6 kinase, a signaling molecule downstream of AKT. Since the above experiment suggests that activation of IGF-1R may be responsible for AKT activation, we wanted to determine if activation of S6K is also dependent on IGF-1R activation. In Figure 7, we show that Cyclolignan PPP inhibits the activation of S6 kinase after androgen withdrawal, confirming an important role of IGF-1R activity in the activation of the AKT-S6K pathway in LNCaP cells after androgen withdrawal.

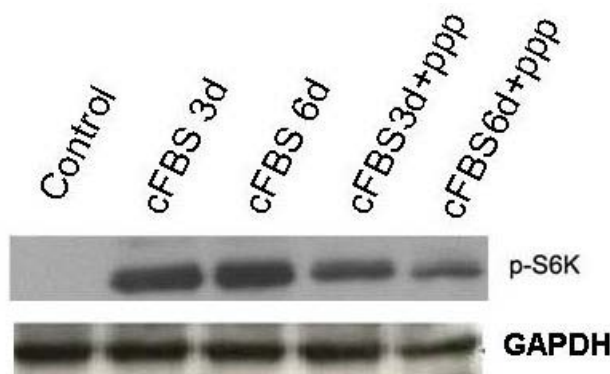


Fig. 7. Cyclolignan PPP inhibits the activation of S6K after androgen deprivation. LNCaP cells were cultured normally (control) or in charcoal-treated FBS (cFBS) in the absence or presence of Cyclolignan PPP. Equal amounts of proteins were resolved by SDS-PAGE and immunoblotted with an anti-s6K antibody. Androgen deprivation for 3 and 6 days increases tyrosine phosphorylation of IGF-1R, which is inhibited by IGF-1R inhibitor cyclolignan PPP

The above experiments suggest that activation of IGF-1R after androgen withdrawal is necessary for the activation of AKT-S6K pathway in LNCaP cells. Since we have shown previously that activation of AKT is required for neuroendocrine differentiation of LNCaP cells after androgen withdrawal (2), we hypothesize that activation of IGF-1R may also be required for neuroendocrine differentiation after androgen withdrawal. Thus, we cultured LNCaP cells normally or in charcoal-treated FBS for 3 and 6 days, respectively in the absence or presence of Cyclolignan PPP. Neuroendocrine differentiation was studied by a quantitative real-time PCR to determine the levels of mRNA for two neuroendocrine markers, neuron-specific enolase (NSE) and Chromogranin A (CgA). As shown in Figure 8, androgen deprivation induces neuroendocrine differentiation of LNCaP cells with increased expression of NSE and CgA. Neuroendocrine differentiation is inhibited by Cyclolignan PPP, suggesting the IGF-1R activity is required for this process.

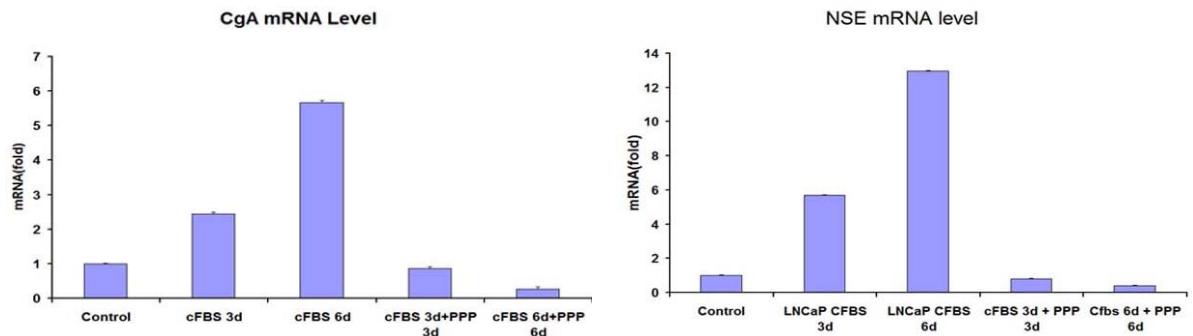


Fig. 8. Fig. 7. Cyclolignan PPP inhibits neuroendocrine differentiation of LNCaP cells after androgen deprivation. LNCaP cells were cultured normally (control) or in charcoal-treated FBS (cFBS) in the absence or presence of Cyclolignan PPP. Androgen deprivation for 3 and 6 days increases the expression of neuroendocrine markers NSE and CgA, which is inhibited by IGF-1R inhibitor cyclolignan PPP

## Conclusions from experiments performed for task 2A:

The above experiments establish IGF-1 receptor as a critical player in androgen-withdrawal-induced neuroendocrine differentiation of prostate cancer cell line LNCaP.

## Task 2B. The mechanism of action of PTP1B in regulating the signaling of IGF-1R.

To accomplish this task, we will use LNCaP cells that stably overexpress PTP1B to determine if IGF-1R and its downstream signaling pathway are activated. We will also study if expression of a dominant negative mutant PTP1B inhibits activation of IGF-1R and its downstream signaling pathway upon androgen withdrawal

## Accomplishments:

1. We have previously shown that expression of a protein tyrosine phosphatase, PTP1B, induces neuroendocrine differentiation of LNCaP cells (3). Since the above experiments in task 2A suggest that androgen deprivation of LNCaP can activate IGF-1R leading to neuroendocrine differentiation, we wanted to determine if the effect of PTP1B is also



mediated through IGF-1R. We have established stable cell lines expressing wild-type PTP1B and a mutant PTP1B which has lost its enzymatic activity (3). Parental LNCaP cells and LNCaP expressing wild type PTP1B (LNCaP/PTP1B) or the mutant PTP1B (LNCaP/PTP1BM) were cultured normally or in charcoal-treated FBS (androgen-deprived). Equal amounts of protein were immunoprecipitated by an anti-IGF-1R antibody and immunoblotted by an anti-phospho-tyrosine antibody. As shown in Figure 9, expression of either the wild-type or the mutant PTP1B increases the tyrosine phosphorylation of IGF-1R and the levels of tyrosine phosphorylation are further increased after androgen withdrawal. Therefore, it appears that PTP1B expression activates IGF-1R but this effect is further enhanced by androgen deprivation. Another important conclusion is that this effect appears to be independent of its protein tyrosine phosphatase enzymatic activity.

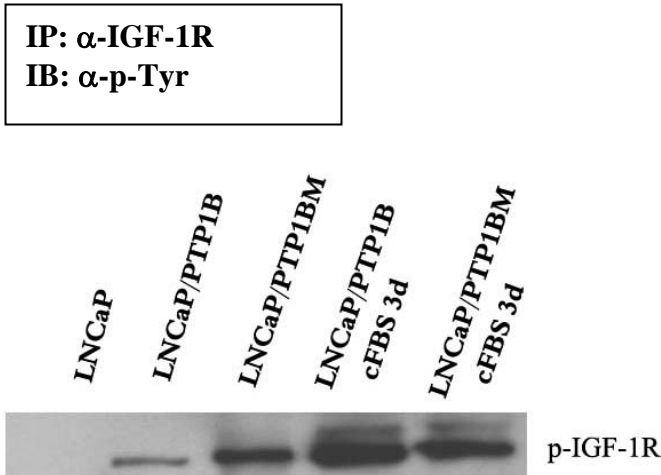


Fig. 9. IGF-1R is activated in LNCaP cells expressing the wild and the mutant PTP1B. LNCaP cells, LNCaP/PTP1B or LNCaP/PTP1BM were cultured normally or in charcoal-treated FBS. Equal amounts of protein were immunoprecipitated by an anti-IGF-1R antibody and immunoblotted by an anti-phospho-tyrosine antibody. Tyrosine phosphorylation of IGF-1R is increased when either the wild-type or the mutant PTP1B is expressed. The level of phosphorylation is further increased after androgen withdrawal for 3 days.

We also studied the level of phosphorylation of AKT, a signaling molecule downstream of IGF-1R. Similarly, we found that expression of the wild type or the mutant PTP1B increases phosphorylation of AKT which is not dramatically changed by androgen deprivation (Fig. 10)

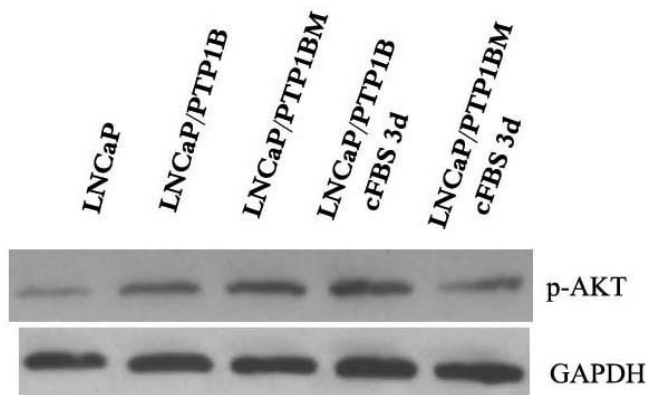


Fig. 10. Expression of the wild and the mutant PTP1B increases the phosphorylation of AKT. LNCaP cells, LNCaP/PTP1B or LNCaP/PTP1BM were cultured normally or in charcoal-treated FBS. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted by an anti-phospho-AKT antibody. Expression of PTP1B (wild type or the mutant) increases the phosphorylation of AKT. The level of phosphorylation is not further increased after androgen withdrawal for 3 days.

To determine if the expression of PTP1B or its mutant may affect IGF-1R signaling, we add IGF-1 (100 ng/ml) to normally cultured LNCaP cells, LNCaP/PTP1B cells and LNCaP/PTP1BM cells for 6 days and study the activation of the key signaling molecules downstream of IGF-1R. Consistent with the results shown above, expression of either the wild type or the mutant PTP1B increases phosphorylation of AKT. IGF-1 treatment does not change AKT phosphorylation in these cells significantly, likely due to the fact that AKT is already highly phosphorylated (Fig. 11).

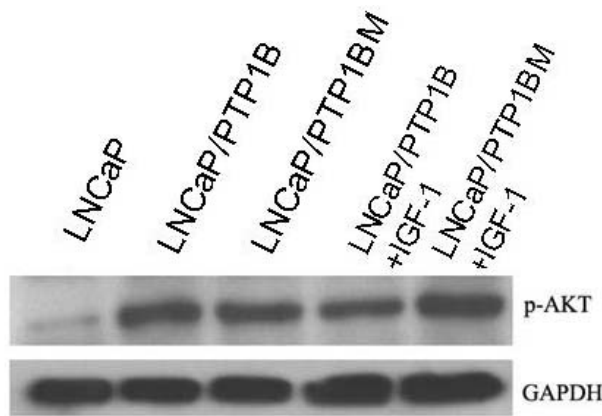


Fig. 11. IGF-1 treatment does not further increase phosphorylation of AKT in LNCaP cells expressing wild type or mutant PTP1B. LNCaP cells, LNCaP/PTP1B cells (-/+ 100ng/ml of IGF1 for 6 days) and LNCaP/PTP1BM cells (-/+ 100 ng/ml of IGF-1 for 6 days) were harvested and equal proteins were resolved by SDS-PAGE and immunoblotted with an anti-pAKT antibody. LNCaP cells expressing wild-type and mutant PTP1B have increased AKT phosphorylation which is not further enhanced by IGF-1 treatment.

To confirm the above finding on the status of AKT phosphorylation, we studied the phosphorylation of S6 kinase, a downstream signaling molecule of AKT. We obtained similar results, that is, expression of either the wild type or the mutant PTP1B increases phosphorylation of S6K. IGF-1 treatment does not change S6K phosphorylation in these cells significantly, likely due to the fact that S6K is already highly phosphorylated (Fig. 12).

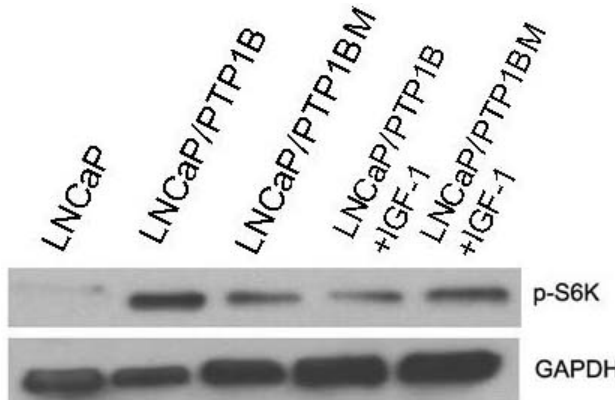


Fig. 12. IGF-1 treatment does not further increase phosphorylation of S6K in LNCaP cells expressing wild type or mutant PTP1B. LNCaP cells, LNCaP/PTP1B cells (-/+ 100ng/ml of IGF1 for 6 days) and LNCaP/PTP1BM cells (-/+ 100 ng/ml of IGF-1 for 6 days) were harvested and equal proteins were resolved by SDS-PAGE and immunoblotted with an anti-pS6K antibody. LNCaP cells expressing wild-type and mutant PTP1B have increased S6K phosphorylation which is not further enhanced by IGF-1 treatment.

Therefore, we have observed activation of IGF-1R upon the expression of PTP1B, which may be the upstream event leading to activation AKT signaling pathway. The function of PTP1B in this process appears to be independent of its enzymatic activity as mutant PTP1B without tyrosine phosphatase activity gives similar results.

As a support to the above interpretation, we have also observed that the tyrosine phosphorylation of IRS-2 is also increased in LNCaP cells expressing either wild-type or mutant PTP1B, which, similarly, is not enhanced by IGF-1 treatment. (Fig. 13).

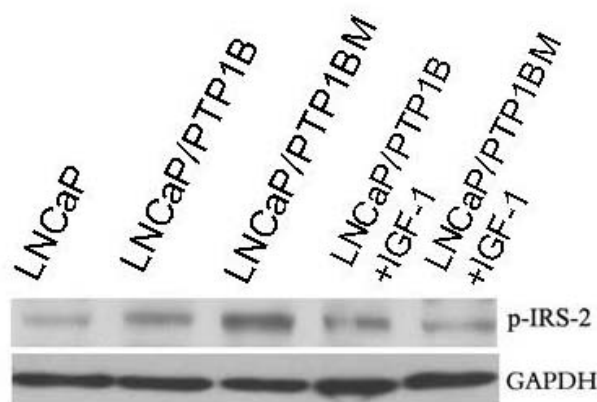


Fig. 13. IGF-1 treatment does not further increase tyrosine phosphorylation of IRS-2 in LNCaP cells expressing wild type or mutant PTP1B. LNCaP cells, LNCaP/PTP1B cells (-/+ 100ng/ml of IGF1 for 6 days) and LNCaP/PTP1BM cells (-/+ 100 ng/ml of IGF-1 for 6 days) were harvested and equal proteins were resolved by SDS-PAGE and immunoblotted with an anti-pIRS-2 antibody. LNCaP cells expressing wild-type and mutant PTP1B have increased IRS-2 phosphorylation which is not further enhanced by IGF-1 treatment.

### Conclusions from experiments performed for task 2B:

In conclusion, we have shown that IGF-1R is activated by the expression of PTP1B in LNCaP cells, which may be responsible for the activation of AKT signaling pathway. In addition, our results suggest that this activity of PTP1B appears to be independent of its tyrosine phosphatase enzymatic activity.

### Task 3. The function of PTP1B in androgen receptor signaling

Work related to this task will be performed later.

### Additional significant findings that were not anticipated in the original proposal

Our ultimate goal is to determine the molecular mechanisms that are responsible for the recurrence of PC after hormonal therapy. We hypothesize that NE tumor cells, being AR negative, are resistant to hormonal therapy that targets AR signaling and will survive hormonal therapy, which may lead to tumor recurrence. As mentioned in last year's annual progress report, as we were studying the function of NE cells in PC, we started paying more attention to the cancer stem cells theory and we have since unequivocally proven in human PC tissue that NE tumor cells are the CD44+ cells. We have previously reported that NE tumor cells are quiescent and do not express AR and PSA (1). Our results, in combination with the report by Patrawala et al (4) showing that CD44 expression identifies cancer stem cells, strongly suggest that NE tumor cells of PC may represent the PC stem cells. A manuscript describing this finding has been accepted for publication recently (5) and attached.

We have extended this finding to small cell carcinoma of the prostate, a prostate tumor composed entirely of neuroendocrine cells. We showed that in such tumors, the tumor cells are positive for CD44, a feature that distinguishes it from small cell carcinoma of other origins. In fact, we have identified the first marker that shows organ specificity. Our manuscript was published recently (6)

### KEY RESEARCH ACCOMPLISHMENTS:

1. We have shown that IGF-1 receptor may be a critical player in androgen-withdrawal-induced neuroendocrine differentiation of prostate cancer cell line LNCaP.
2. We have shown that IGF-1 receptor is activated by the expression of a protein tyrosine phosphatase PTP1B in LNCaP cells, which may be responsible for the activation of AKT signaling pathway. In addition, our results suggest that this activity of PTP1B appears to be independent of its tyrosine phosphatase enzymatic activity.

## **REPORTABLE OUTCOMES:**

### Manuscripts:

1. Simon RA, di Sant'Agnese PA, Huang L-S, Xu H, Yao JL, Yang Q, Liang S, Liu, J, Lee R, Cheng L, Oh WK, Palapattu G, Wei J, and **Huang J**. CD44 Expression is a Feature of Prostatic Small Cell Carcinoma and Distinguishes it from its Mimickers. **Hum Pathol** 2009;40:252-8
2. Palapattu GS, Wu C, Silvers CR, Martin HB, Williams K, Salamone L, Bushnell T, Huang L-S, Yang Q and **Huang J**. Selective expression of CD44, a putative prostate cancer stem cell marker, in neuroendocrine tumor cells of human prostate cancer. **Prostate** 2009 (in press)

### Presentations:

1. Departmental Seminar Speaker, Department of Pharmacology & Experimental Therapeutics, University of Maryland School of Medicine, June 2008 (Neuroendocrine cells in prostate cancer).
2. Invited Speaker, 15<sup>th</sup> Annual Meeting of the Chinese Urological Society, Kunming, China, September 2008 (Neuroendocrine cells in prostate cancer).

### Funding applied for based on work supported by this award:

#### Received:

1. University of Rochester CTSI Laboratory Support Fund: Received in 2008 to support a tissue microarray study on gene regulation in LNCaP cells after activation of CXCR2 by IL-8 in LNCaP cells
2. UCLA SPORE in Prostate Cancer Development Research Grant: Received in January 2009 to study the function of neuroendocrine cells in prostate cancer recurrence after hormonal therapy

#### Pending:

1. Prostate Cancer Foundation (Huang): The function of neuroendocrine cells in prostate cancer
2. New York State Stem Cell Fund (Palapattu/Huang): The function of neuroendocrine cells in prostate cancer
3. New York State Stem Cell Fund (Zeng and Huang): Targeting neuroendocrine cells in prostate cancer

4. NIH Director's New Innovator Award (Huang): The function of neuroendocrine cells in prostate cancer

Employment received based on experience supported by this award:

In the fall of 2008, I was recruited by UCLA David Geffen School of Medicine to be Professor of Pathology and Director of Urologic Pathology based on experience supported by the award.

**CONCLUSION:**

Neuroendocrine cells, although comprising a small population of the prostate cancer cells, may be responsible for tumor recurrence after hormonal therapy as they do not express androgen receptor and are resistant to therapy. Our work demonstrates that there is a complex network of multiple signaling pathways that maintains the unique phenotype of the neuroendocrine cells, and coordination and cross-talk of the different pathways may be essential. We have shown that IGF-1 receptor may be a critical player in androgen-withdrawal-induced neuroendocrine differentiation of prostate cancer cell line LNCaP. We have also shown that IGF-1 receptor is activated by the expression of a protein tyrosine phosphatase PTP1B in LNCaP cells, which may be responsible for the activation of AKT signaling pathway. In addition, our results suggest that this activity of PTP1B appears to be independent of its tyrosine phosphatase enzymatic activity. Prostate cancer, in its localized form, can be effectively treated by surgery or radiation therapy. The currently adopted method to treat advanced and metastatic cancer is hormonal therapy. The therapy is effective initially but fails eventually in every single patient. We have demonstrated that neuroendocrine cells, although comprising a small population of the cancer, may be resistant to hormonal therapy and responsible for tumor recurrence. Therefore, to achieve a cure, neuroendocrine cells should be targeted. We have also demonstrated complex signaling networks in maintaining the neuroendocrine phenotype of the tumor cells which are potential therapeutic targets.

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6. Simon RA, di Sant'agnese PA, Huang LS, Xu H, Yao JL, Yang Q, Liang S, Liu J, Yu R, Cheng L, Oh WK, Palapattu GS, Wei J, Huang J. CD44 expression is a feature of prostatic small cell Carcinoma and Distinguishes it from its Mimickers. *Hum Pathol* 2008.

#### **APPENDICES:**

- 1 Simon RA, di Sant'Agnese PA, Huang L-S, Xu H, Yao JL, Yang Q, Liang S, Liu, J, Lee R, Cheng L, Oh WK, Palapattu G, Wei J, and **Huang J**. CD44 Expression is a Feature of Prostatic Small Cell Carcinoma and Distinguishes it from its Mimickers. **Hum Pathol** 2009;40:252-8
- 2 Palapattu GS, Wu C, Silvers CR, Martin HB, Williams K, Salamone L, Bushnell T, Huang L-S, Yang Q and **Huang J**. Selective expression of CD44, a putative prostate cancer stem cell marker, in neuroendocrine tumor cells of human prostate cancer. **Prostate** 2009 (in press)

**Original contribution**

# CD44 expression is a feature of prostatic small cell Carcinoma and Distinguishes it from its Mimickers<sup>☆</sup>

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Received 19 June 2008; revised 21 July 2008; accepted 23 July 2008

**Keywords:**

Prostate;  
Adenocarcinoma;  
Small cell carcinoma;  
CD44;  
Immunohistochemistry

**Summary** Small cell neuroendocrine carcinoma of the prostate is a rare variant of prostatic cancer that shares morphologic similarity with prostatic adenocarcinoma of Gleason 5 pattern. It has also been considered morphologically and immunohistochemically indistinguishable from small cell neuroendocrine carcinomas of other origins. CD44 is a cell-surface molecule proposed to identify cancer stem/progenitor cells in prostate cancer. We performed immunohistochemical study for CD44 expression in 11 cases of prostatic small cell neuroendocrine carcinoma and compared its patterns of expression with 73 cases of prostatic adenocarcinoma and 47 cases of small cell neuroendocrine carcinomas of other organs. Strong and diffuse membrane staining for CD44 was observed in 100% of the prostatic small cell neuroendocrine carcinomas. In conventional adenocarcinomas of the prostate, positive staining was only seen in rare, scattered tumor cells; and CD44 staining was negative in most of the small cell neuroendocrine carcinomas of nonprostate origin. The difference in CD44 expression between small cell neuroendocrine carcinomas of the prostate and those of other organs are statistically significant ( $P < .001$ ). Our study demonstrates the utility of immunohistochemical staining for CD44 in distinguishing prostatic small cell neuroendocrine carcinoma from its mimickers including prostatic adenocarcinoma of Gleason 5 pattern and small cell neuroendocrine carcinomas of other organs. CD44 is the first marker that shows a high degree of tissue/organ specificity for small cell neuroendocrine carcinomas. Because CD44 is a putative marker of prostate cancer stem cells, the strong and diffuse

<sup>☆</sup> J. Huang is supported by the Department of Defense Prostate Cancer Research Program (PC061456) and American Cancer Society (RSG-07-092-01-TBE).

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expression of CD44 and the lack of expression of prostate luminal differentiation markers androgen receptor and prostatic specific antigen in prostatic small cell neuroendocrine carcinomas suggest that the tumor cells may retain cancer stem cell features.

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## 1. Introduction

Prostate cancer (PC) is the most common malignancy in men and the second leading cause of cancer-related deaths [1]. Most of the tumors (>90%) in the prostate are adenocarcinomas that recapitulate the morphology of prostatic acini. A minority of the prostatic epithelial malignancies are variant forms including ductal-type adenocarcinoma, mucinous (colloid) carcinoma, signet ring cell carcinoma, and small cell (neuroendocrine) carcinoma (SCNC) [2]. Among these variants, SCNCs possess many unique histologic, ultrastructural, immunohistochemical, and clinical features. Morphologically, these tumors are considered indistinguishable from pulmonary and other extrapulmonary SCNCs with a solid, sheet-like growth pattern, usually with areas of tumor necrosis. Tumor cells are small, with fine chromatin pattern, scant cytoplasm, and nuclear molding. Mitotic figures and crush artifact are frequent findings [3–5].

SCNCs of the prostate are rather rare and account for no more than 1% of all carcinomas of the prostate. Similar to SCNCs of other sites, they are aggressive tumors and often present as locally advanced or metastatic diseases [6]. Occasionally, they are associated with paraneoplastic syndromes [7]. Although they may arise *de novo*, such tumors may also occur as recurrent tumors after hormonal therapy for conventional adenocarcinomas of the prostate [8,9]. SCNC may be present either as a pure form or as a component of mixed tumors, which also contain conventional adenocarcinoma. Immunohistochemically, they share similarities with SCNCs from other organs including dot-like perinuclear staining for cytokeratin cocktail and positivity for thyroid transcription factor 1 (TTF-1) and neuroendocrine markers chromogranin A, synaptophysin, and neuron specific enolase (NSE) [3,4]. In addition, they generally do not express markers of prostatic luminal differentiation such as androgen receptor (AR), prostatic specific antigen (PSA), and prostate acid phosphatase (PAP) [3–5].

CD44 is a single-chain glycoprotein with a conserved N-terminal extracellular domain, a nonconserved membrane proximal region, a conserved transmembrane domain, and a conserved cytoplasmic tail. There are numerous isoforms of CD44 due to alternative splicing of CD44 messenger RNA, and the variability in CD44 structure is further increased by N- and O-glycosylation as well as the attachment of glycosaminoglycans (eg, heparan sulfate, chondroitin sulfate [10]). CD44 is a major cell-surface receptor for hyaluronic acid and mediates epithelial cell adhesion by its involvement in cell-cell and cell-matrix

interactions [11]. The CD44 isoforms are also implicated in cell migration and tumor progression [12], and their expression levels reportedly have prognostic value in certain malignancies [13–17].

CD44 has attracted significant attention recently because of its potential as a cancer stem cell marker for certain solid tumors. Al-Hajj et al [18] showed that the breast cancer stem cells have the surface markers CD44<sup>+</sup>/CD24<sup>−</sup>/lowESA<sup>+</sup>. In PC, Collins et al [19] showed that the CD44<sup>+</sup>/α2β1<sup>hi</sup>/CD133<sup>+</sup> cells have cancer stem cell properties. However, in 2 comprehensive studies, Tang's group demonstrated that CD44 expression by itself identifies the putative stem cells of PC [20,21]. In this article, we report our studies on the expression of CD44 in benign prostate, prostatic adenocarcinoma, prostatic SCNC, as well as SCNCs arising in other organs.

## 2. Materials and methods

The study was approved by the University of Rochester Research Subject Review Board, and all patients' identifiers were removed to protect confidentiality. A total of 131 cases were studied, including 73 cases of prostatic adenocarcinoma built into a tissue microarray (TMA) and 58 cases of SCNCs of various origins. There were 11 cases of prostatic SCNCs (taken from a cohort used in a previous publication [3]) and 47 cases of SCNCs of other origins including lung (11 cases), female genital organs (cervix, lower uterine segment, vagina and ovary, 14 cases), urinary bladder (10 cases), head and neck (6 cases), stomach (3 cases), and pancreas (3 cases).

The prostate TMA was constructed as previously described [22]. Briefly, prostatectomy specimens were reviewed, and areas containing prostate adenocarcinoma were marked for sampling. Tumors with Gleason patterns 2 and 3 were classified as low grade and those with Gleason patterns 4 and 5 were classified as high-grade tumors. Two to three cores per sample, measuring 0.6 mm in diameter, were obtained from selected regions in each donor paraffin block and transferred to a recipient paraffin block. For SCNCs, regular histologic sections were used in the study.

Immunohistochemical staining was performed using a monoclonal anti-CD44 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, sc-7297, 1:1000 dilution), which recognizes CD44s and all of its isoforms. The detailed staining procedure has been described previously [22]. Positive cells showed



strong membrane staining pattern. Statistical analysis was performed using  $\chi^2$  tests with the assistance of a biostatistician (L.-S. Huang).

### 3. Results

#### 3.1. Expression of CD44 in benign prostate tissue

Benign prostate contains 2 major types of epithelial cells, the luminal secretory cells and the basal cells that surround the luminal cells. Immunohistochemical study showed that the luminal secretory cells were negative for CD44 expression, whereas the staining was strong and diffuse in all basal cells, consistent with previous studies reported in the literature [3-5] (Fig. 1A).

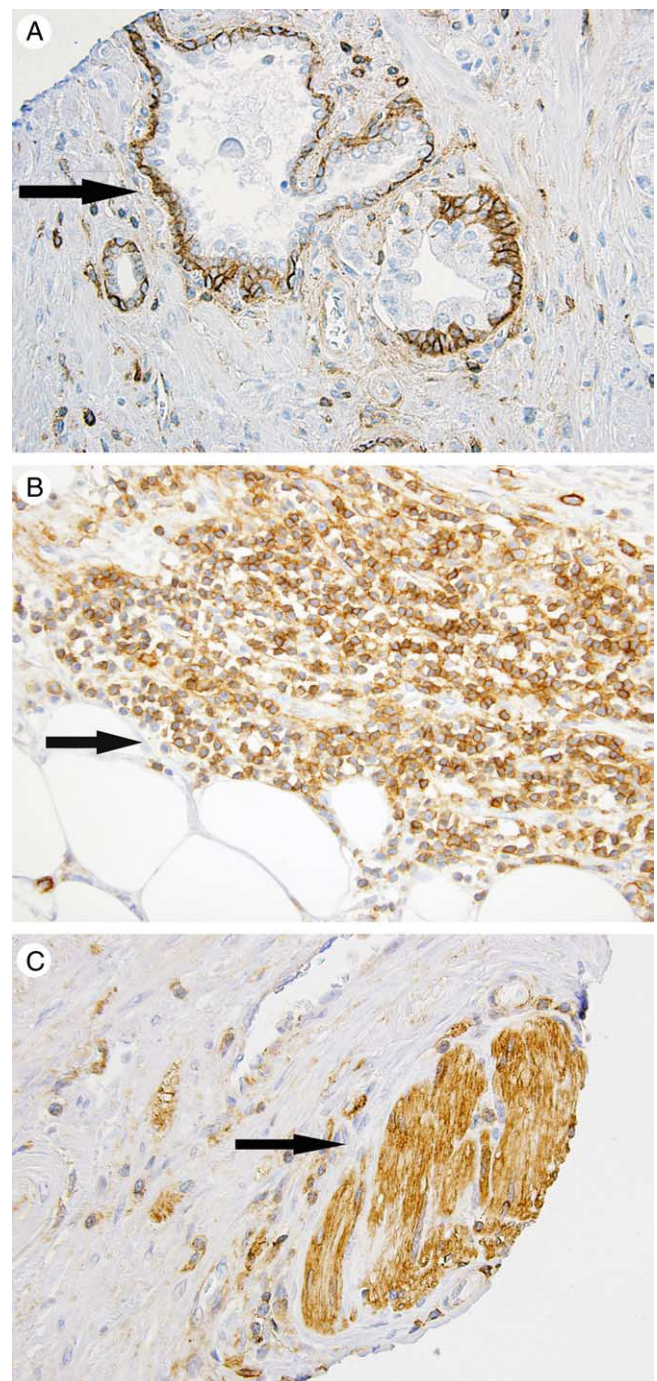
The stromal cells of the prostate were negative for CD44 expression, but interestingly, CD44 expression was strong and diffuse in lymphocytes (Fig. 1B) and nerves (Fig. 1C).

#### 3.2. Expression of CD44 in prostatic adenocarcinoma

Adenocarcinoma of the prostate consists of cancerous epithelial cells with luminal differentiation (expression of AR, PSA, and PAP) but no basal cells. Immunohistochemical study using sections from the TMA showed that most of the cancer cells in each core were negative for CD44 expression (Fig. 2). However, there were occasional scattered individual tumor cells or small nests of tumor cells that stained strongly in a distinct membranous pattern (Fig. 2). We performed a quantitative analysis of all the cores. We showed that, among the 40 cases of low-grade tumors, 19 cases (47.5%) had no positive cells, 17 cases (42.5%) contained up to 0.5% of positive cells, 4 cases (10%) contained between 0.5% and 1% of positive cells, whereas no cases (0%) had more than 1% of positive cells. Among the 33 cases of high-grade tumors, 8 cases (24.2%) had no positive cells, 13 cases (39.4%) contained up to 0.5% positive cells, 9 cases (27.3%) had between 0.5% and 1% of positive cells, whereas 3 cases (9.1%) had between 1% and 2% of positive cells.

#### 3.3. Expression of CD44 in SCNCs of the prostate and nonprostate origins

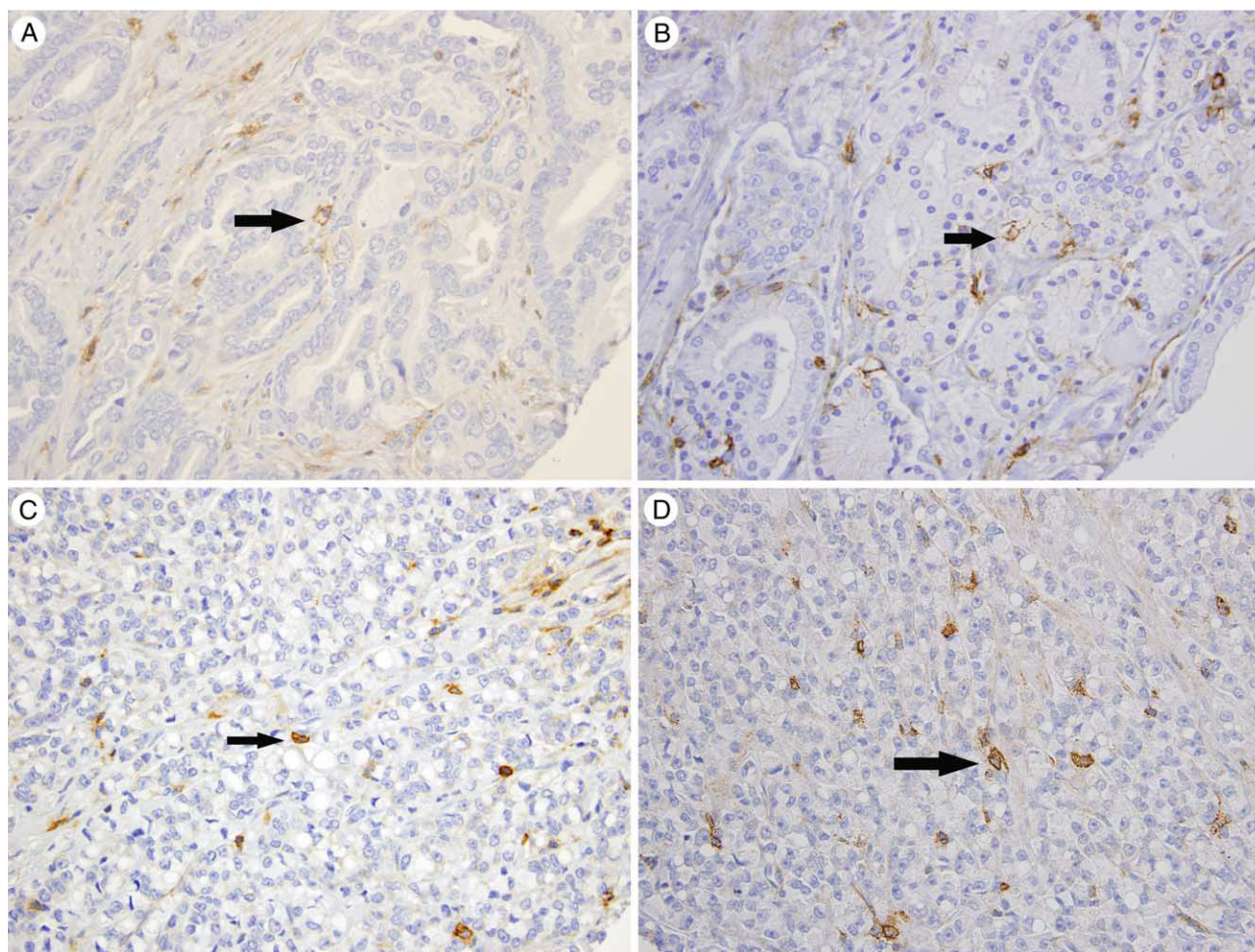
We stained 11 cases of prostatic SCNCs and 47 cases of SCNCs of nonprostatic origin for the expression of CD44. It has been universally accepted that SCNCs have similar light microscopic and ultrastructural morphology and immunohistochemical profile, regardless of tissue of origin. To our surprise, anti-CD44 antibody stained all cells of prostatic SCNCs strongly and diffusely in nearly 100% of the cases. Among the 11 cases, 5 showed positive staining in 100% of the cells, whereas the remaining 6 cases showed positive



**Fig. 1** Immunohistochemical study for CD44 expression in benign prostate tissue. A, Positive staining in basal cells (arrow). B, Positive staining in lymphocytes (arrow). C, Positive staining in a nerve (arrow) (original magnification  $\times 400$ ).

staining in 60-90% of the tumor cells. In contrast, most of the nonprostatic SCNCs were completely negative for CD44. Among the 47 nonprostatic SCNC cases, 4 of 11 cases of pulmonary SCNCs contained positive cells (100%, 2 cases; 90%, 1 case; 10%, 1 case), 3 of 8 cases of uterine cervical SCNCs contained positive cells (90%, 2 cases; 30%, 1 case);





**Fig. 2** Immunohistochemical study of CD44 expression in adenocarcinomas of the prostate. A and B, Focal CD44-positive staining in low-grade tumors (arrow). C and D, Focal CD44-positive staining in high-grade tumors (arrow) (original magnification  $\times 400$ ).

2 of 6 cases of head and neck SCNCs contained positive cells (90% and 10%, respectively), and 1 of 3 cases of pancreatic SCNCs (10%). If 10% positive staining is used as the cutoff value, 100% (11/11) of prostatic SCNCs were positive and only 23% (11/47) of nonprostatic SCNCs were positive. If 20% positive staining is used as the cutoff value, 100% (11/11) of prostatic SCNCs were positive and only 15% (7/47) of nonprostatic SCNCs were positive. With  $\chi^2$  test, the

difference between prostatic SCNC and nonprostatic SCNC was statistically significant ( $P < .001$ ) using either 10% or 20% as the cutoff value. The results are summarized in Table 1, and representative pictures are shown in Fig. 3.

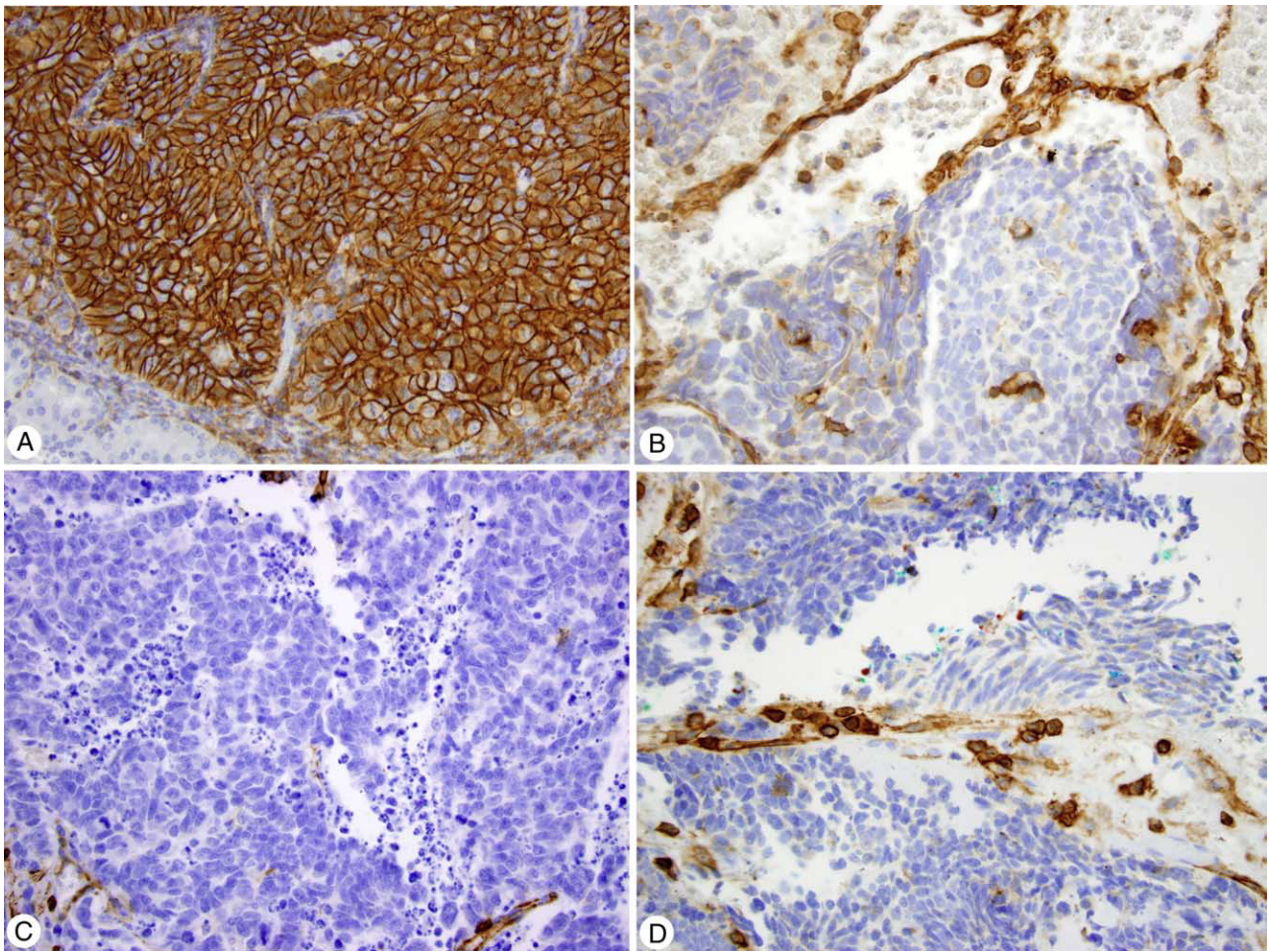
#### 4. Discussion

SCNCs of the prostate are aggressive tumors in comparison to prostatic adenocarcinomas. Some adenocarcinomas recur as SCNCs after hormonal therapy, suggesting that the tumor cells can proliferate in an androgen-deprived environment. Histologically, it is often difficult to distinguish SCNCs of the prostate from Gleason pattern 5 conventional adenocarcinomas of the prostate because they share similar morphologic features including sheet-like growth pattern and no obvious glandular formation [23]. However, this is an important clinical distinction because the 2 tumors are biologically different. High-grade adenocarcinomas express AR and respond well to hormonal therapy that aims to stop androgen production and/or block the function of AR [6,24].

**Table 1** CD44 expression in SCNCs

Tumor origin	Positive ( $\geq 10\%$ )/total	Positive ( $\geq 20\%$ )/total
Prostate	11/11 (100%)	11/11 (100%)
Nonprostate origin	11/47 (23.4%)	7/47 (14.9%)
Lung	4/11	3/11
Head and neck	2/6	1/6
Female genital organs	4/14	3/14
Urinary bladder	0/10	0/10
Stomach	0/3	0/3
Pancreas	1/3	0/3





**Fig. 3** Immunohistochemical study of CD44 expression in SCNCs of prostate and nonprostate origins. A, Diffuse strong membrane CD44 staining in SCNC of the prostate. B to D, Negative CD44 staining in SCNC of the lung (B), cervix (C), and urinary bladder (D). Some positive staining in B to D represents lymphocytes and stromal cells (original magnification  $\times 400$ ).

SCNC of the prostate, on the other hand, often presents with locally advanced or metastatic disease with a life expectancy of a few months [24,25]. In addition, SCNCs do not express AR and as a result do not respond to hormonal therapy [3,4]. Immunohistochemically, adenocarcinomas usually express AR, PSA, and PSAP, whereas SCNCs usually express neuroendocrine markers (chromogranin A, synaptophysin, NSE) and TTF-1 [4], which can be used to distinguish them in difficult cases. However, the immunohistochemical profiles are not consistent and SCNCs can be negative for neuroendocrine markers/TTF-1 and express PSA/PSAP in occasional cases [3,4]. Therefore, morphology remains the gold standard in the diagnosis of SCNC [23]. Unfortunately, morphologic interpretation alone suffers from subjectivity and significant interobserver variability. Here we have shown that in conventional adenocarcinomas, CD44 is positive in only rare scattered tumor cells (up to 1%-2% of tumor cells), whereas in SCNCs of the prostate, the staining is strong and diffuse (60%-100% tumor cells), making CD44 a reliable marker to distinguish the 2 morphologically similar but biologically different tumors.

Another important utility of CD44 staining is in distinguishing SCNC of the prostate from that of other origins. All SCNCs, regardless of origin, have been considered morphologically, ultrastructurally, and immunohistochemically identical. Therefore, when the disease is present locally, it is sometimes difficult to distinguish prostatic SCNC from bladder SCNC, particularly when a SCNC has spread from prostate to bladder or vice versa. Moreover, SCNCs are aggressive diseases that often present with metastasis that makes determination of origin difficult at times, particularly when the primary tumor is not identified or metastatic tumors involve multiple organs. Because CD44 is positive in all SCNCs of the prostate and only in a minority of SCNCs of other organs, strong and diffuse positivity for CD44 in a case of metastatic SCNC of unknown origin in an older man would strongly suggest origin from the prostate. Therefore, in addition to identifying a marker for the differential diagnosis of adenocarcinoma and SCNC of the prostate, our study has also identified the first marker that shows a high degree of tissue/organ specificity for SCNCs.

In the normal prostate, CD44 is expressed in basal cells [17,26,27]. In PC, the pattern of expression of CD44 remains controversial. One study of 109 cases found a complete lack of expression of all CD44 isoforms in most (93%-98%) of primary PCs [28]. However, another study of 74 cases found moderate to high levels of CD44 expression in 60% of primary PCs with 14% of metastases expressing low levels of CD44 [28]. De Marzo et al [29] found significantly reduced CD44 expression in all 94 primary PCs and 48 metastatic PCs and reduced expression correlated with Gleason grade, whereas no correlation between Gleason grade and CD44 expression was found in another study [26]. Furthermore, although it has been reported that CD44 expression is reduced in metastatic PCs [28,29], the CD44<sup>+</sup> PC cells were found to predominate in 2 visceral metastases [30]. In our study using a TMA containing 73 cases of PC, we found only 0% to 2% of positive staining in any core, suggesting that the CD44<sup>+</sup> cells are only a minority of the cancer cells. It would be interesting to study the identity of these CD44<sup>+</sup> cells and their significance in PC biology, particularly because CD44 has been identified as a putative PC stem cell marker [20].

Expression of CD44 in SCNCs of certain organs has been reported. For example, Iczkowski et al [31] has shown that most (25/27 cases) of the SCNCs of the bladder do not express CD44v6, and this can be used to different bladder SCNCs from urothelial carcinomas [32]. Expression of CD44s and CD44v6 were similarly rare in SCNCs of the lung (2/14 tumors) [33]. Among the different subtypes of lung cancer, SCNCs express CD44 least frequently [34-40]. Our results are consistent with the published data and suggest that the consistently strong and diffuse staining of CD44 in almost all tumor cells is a unique feature for prostatic SCNCs.

In addition to being useful in the differential diagnosis of prostatic SCNCs from their mimickers with remarkable sensitivity and specificity, the patterns of expression of CD44 in prostatic SCNCs also have important implications in tumor biology. PC is a hormonally regulated tumor, and the tumor cells are dependent on androgen for proliferation. Localized tumors can be cured by surgery and radiation therapy, but for advanced and metastatic tumors, hormonal therapy, consisting of androgen ablation and antiandrogen, is effective in essentially all patients [41-43]. Unfortunately, the effect of hormonal therapy is temporary and the tumor will eventually recur and enter the androgen-independent (hormone-refractory) stage. Although most studies focus on altered AR signaling, there is increasing evidence that cancer stem cells may be responsible for tumor recurrence after hormonal therapy [20,21]. By definition, PC stem cells are undifferentiated and do not express luminal differentiation markers AR, PSA, and PAP. We hypothesize that the tumor cells in SCNCs of the prostate possess features of cancer stem cells as they express the putative cancer stem cell marker CD44 but not the luminal differentiation markers, which may explain why such tumors are extremely aggressive and unresponsive to hormonal therapy.

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Author Proof

# Selective<sup>Q1</sup> Expression of CD44, a Putative Prostate Cancer Stem Cell Marker, in Neuroendocrine Tumor Cells of Human Prostate Cancer

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**BACKGROUND.** Hormonal therapy is effective for advanced prostate cancer (PC) but the disease often recurs and becomes hormone-refractory. It is hypothesized that a subpopulation of cancer cells, that is, cancer stem cells (CSCs), survives hormonal therapy and leads to tumor recurrence. CD44 expression was shown to identify tumor cells with CSC features. PC contains secretory type epithelial cells and a minor population of neuroendocrine cells. Neuroendocrine cells do not express androgen receptor and are quiescent, features associated with CSCs. The purpose of the study was to determine the expression of CD44 in human PC and its relationship to neuroendocrine tumor cells.

**METHODS.** Immunohistochemistry and immunofluorescence were performed to study CD44 expression in PC cell lines, single cells from fresh PC tissue and archival tissue sections of PC. We then determined if CD44+ cells represent neuroendocrine tumor cells.

**RESULTS.** In human PC cell lines, expression of CD44 is associated with cells of NE phenotype. In human PC tissues, NE tumor cells are virtually all positive for CD44 and CD44+ cells, excluding lymphocytes, are all NE tumor cells.

**CONCLUSIONS.** Selective expression of the stem cell-associated marker CD44 in NE tumor cells of PC, in combination with their other known features, further supports the significance of such cells in therapy resistance and tumor recurrence. *Prostate* 00: 1–12, 2009.

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**KEY WORDS:** prostate cancer; neuroendocrine cell; CD44; cancer stem cell

## INTRODUCTION

Prostate cancer (PC) is the most commonly diagnosed cancer and the second leading cause of cancer-related mortality [1]. Multiple options exist for the treatment of organ-confined PC. The primary treatment of choice for advanced/metastatic PC, however, is hormonal therapy [2], consisting of androgen ablation and/or inhibition of androgen action with anti-androgens. Although most patients initially respond to this therapy, the tumor commonly recurs and enters an

Grant sponsor: Department<sup>Q2</sup> of Defense Prostate Cancer Research Program; Grant number: PC073121; Grant sponsor: AUA Foundation/Astellas Rising Stars in Urology Award; Grant sponsor: American Cancer Society; Grant number: RSG-07-092-01-TBE; Grant sponsor: Department of Defense Prostate Cancer Research Program; Grant number: PC061456

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Received 24 October 2008; Accepted 2 January 2009

DOI 10.1002/pros.20928

Published online 00 month 2009 in Wiley InterScience (www.interscience.wiley.com).

androgen-independent (hormone-refractory) stage for which no durable effective therapy is currently available.

Cancer cells within a given tumor were once considered homogeneous, a situation wherein each cell would have equal malignant potential. Data over the past decade, however, have challenged this hypothesis and established that a hierarchy often exists among tumor cells within a given cancer [3]. In vitro and in vivo assays in hematopoietic cancers as well as breast, brain and colon cancer have shown that only a minor subpopulation (typically 1–2%) of tumor cells possesses the ability to self-renew and recreate the entire tumor, inclusive of all cell types [4]. Such “tumor initiating” cells are termed cancer stem cells (CSCs) [5].

Unlike the bulk cancer cells, CSCs do not express differentiation markers and are typically quiescent. As a result, they may be resistant to traditional therapies that depend on continuous cell cycle activity, such as chemotherapy and radiation. The CSC model predicts that potential CSCs within PC are quiescent and do not express the luminal differentiation markers androgen receptor (AR) and prostate specific antigen (PSA) [6–8]. Therefore, these cells are likely androgen-independent and should survive androgen ablation therapy, leading to tumor recurrence [9]. To date, the critical experiment demonstrating the identification of prostate CSCs from primary human tissue with subsequent illustration that the proffered CSC is tumor-initiating in vivo has not been reported. Nonetheless, many groups have reported potential markers that may be associated with prostate CSCs, including the cell surface markers CD44, integrin  $\alpha\beta 1$ , CD133, CXCR4 and breast cancer resistance protein (BCRP) [10–16] as well as cytokeratin 5/18 double positive intermediate cells [17,18] and the side population of cells [19].

In a comprehensive in vitro and in vivo study using cell lines and xenograft tumor models, Patrawala et al. [20] provided compelling evidence that CD44 expression is associated with stem/progenitor cells of PC. They found a general correlation between the proportion of CD44+ cells and tumorigenicity in PC cell lines, with the highly aggressive, androgen-independent PC3 cells and DU145 cells containing more CD44+ cells than the less aggressive, androgen-dependent LNCaP cells. CD44+ cells had higher clonogenicity and tumorigenicity and also expressed higher levels of stem cell-associated genes than CD44– cells. In addition, the authors noted that CD44+ cells did not express AR, while AR was exclusively detected in the CD44– cell population. Importantly, CD44+, AR–, PC cells were capable of generating CD44–, AR+ tumor cells in in vitro and in vivo assays [20]. These results have provided strong evidence that CD44 is associated

with stem/progenitor cells in PC. Interestingly, in a landmark report, Leong et al. [21] showed that a single cell expressing CD44 as well as a few other stem cell markers can be used to generate mouse prostate. Expression of CD44, however, has not been studied in detail in human PC tissue. If CD44 expression is associated with human prostate CSCs, one might expect that CD44+ tumor cells would be scattered among the more abundant bulk tumor cells that possess features of luminal differentiation including expression of AR and PSA.

It is well established that PC is histologically heterogeneous. The majority of malignant cells are of the secretory type epithelial cells that express AR and secrete PSA. Notably, every case of PC also contains a minor population of cells that have neuron-like morphology and produce biogenic amines and neuropeptides. These cells have been termed neuroendocrine (NE) cells and they reside in the basal layer in benign prostate acini. We and others have characterized these NE cells in PC and shown that unlike the bulk secretory type tumor cells, the NE tumor cells are quiescent and do not express AR or PSA [22–24]. Several groups, including our own, have proposed that these NE cells may be resistant to hormonal therapy and therefore responsible for tumor recurrence following androgen ablation (reviewed in Refs. [25–27]). Here, we report our results showing that the putative CSC marker CD44 is selectively expressed in NE tumor cells of PC, further supporting the importance of such cells in therapy resistance and tumor recurrence and raising interesting questions about the relationship of the NE tumor cells to the elusive PC stem cell.

## MATERIALS AND METHODS

### Established Cell Lines

PC-3 (CRL-1435), DU145 (HTB-81), and LNCaP (CRL-1740) cells were obtained from American Type Culture Collection (ATCC; Manassas, VA). All cell lines were routinely maintained in RPMI 1640 (Invitrogen Corp., Carlsbad, CA) containing Penn-Strep mix (Invitrogen Corp.) and 10% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA).

### Fresh Human Surgical Samples

Fresh human prostate tissue was obtained from patients undergoing radical prostatectomy, in accordance with the protocol approved by the University of Rochester Research Subjects Review Board. Upon removal, fresh prostate tissue was cut into 1 mm cubes using sterile disposable scalpels. After washing in RPMI the tissue was incubated in a CO<sub>2</sub> tissue culture incubator overnight in 112 U/ml hyaluronidase (Sigma

H-3506) and 250 U/ml collagenase 1 (Worthington Biochemical MIE4816). The resultant single cell suspension was neutralized by repeated washing in RPMI/5% FCS followed by resuspension in FACS buffer (1% FBS in D-PBS [Invitrogen Corp.], 0.01% DNase, Sigma, St. Louis, MO). All samples were filtered through a 100 µm cell strainer prior to staining.

#### **Tissue Microarray: Immunohistochemistry and Immunofluorescence**

The prostate TMA was constructed as previously described [28]. Briefly, 73 prostatectomy specimens were reviewed and areas containing prostate adenocarcinoma were marked for sampling. Tumors ranged from Gleason patterns 2 to 5. Two to three cores per samples, measuring 0.6 mm in diameter, were obtained from selected regions in each donor paraffin block and transferred to a recipient paraffin block and the resulting block contained a total of 200 cores. A section was obtained from the TMA for H&E staining as quality control and unstained sections were used for immunohistochemical and immunofluorescence staining.

The procedure for immunohistochemical staining has been described in detail previously [28]. The TMA sections were stained with a mouse monoclonal antibody against chromogranin A (Chemicon International, Inc., Temecula, CA, Clone 2H10, used at 1:1,000), and a rat monoclonal antibody against CD44 (eBioscience, San Diego, CA., Clone IM7, used at 1:1,000). Paraffin embedded tissues were sectioned at 5 µm thickness and antigen retrieval was performed with pre-heated (95–99°C) Citrate Buffer, pH 6.1 (DakoCytomation, Carpinteria, CA) in a Black and Decker steamer (Shelton, CT, Model HS800) for 30 min. The sections were incubated with the primary antibodies at room temperature for 60 min (CD44) or 45 min (chromogranin A), followed by incubation for 30 min with the link antibody (rabbit or mouse) - labeled polymer-HRP (Envision Plus System, DakoCytomation). Slides were developed with AEC+ (DakoCytomation) and counterstained in Modified Mayers Hematoxylin.

For immunofluorescence staining of the TMA section, Antigen retrieval was performed as described above. Anti-CD44 (same source as above, used at 1:200), anti-CD45 (Dako North America, Inc., Carpinteria, CA; M0701, 1:100), and anti-chromogranin A (Dako; A0430, 1:1,000) antibodies were incubated with the TMA slide overnight at room temperature. The slide was then incubated with secondary antibodies (goat anti-rat IgG FITC [Invitrogen Corp.; 62-9511, 1:200], Alexa Fluor 546 goat anti-mouse [Invitrogen Corp.; A-11003, 1:200], and Alexa Fluor 633 F(ab)<sub>2</sub> fragment

of goat anti-rabbit [Invitrogen Corp.; A-21072, 1:200]) for 40 min at room temperature. The slide was mounted with a coverslip using Vectashield HardSet Mounting Medium with DAPI (Vector; H-1500). Tissue cores were photographed individually with a Leica TCS SP Spectral Confocal microscope. Subsequently, the coverslip was removed and the TMA stained with H&E. The H&E-stained tissue cores were then photographed with a Leica DM5000 B microscope. Cancerous areas in each core were marked by a pathologist (JH) and the nuclei manually marked in each digital image and counted using the particle analysis feature of NIH ImageJ software (<http://rsb.info.nih.gov/ij/>). Marked cells in cancerous regions were examined for fluorescence in the corresponding confocal images, and the number of positive cells recorded.

#### **Quantitative RT-PCR**

Detailed method has been described previously [29]. Total RNA was isolated from cells with RNeasy<sup>®</sup> Kit (Qiagen) according to the manufacturers instructions. RNA was reverse transcribed by Transcriptor reverse transcriptase (Roche, Germany) with random hexamers (Promega). The following specific forward and reverse primers were used: for NSE, 5'-AGCTGC CCCTGCCTTAC-3' and 5'-GAGACAAACAGCGTTA CTTAG-3'; for chromogranin A, 5'-GCGGTGGAAG AGCCATCAT-3' and 5'-TCTGTGGCTTCACCACTT TTCTC-3'; for β-actin, 5'-GCCGGAAATCGTGCGT GACATT-3' and 5'-GATGGAGTTGAAGGTAGTTTC GTG-3'.

Real time PCR was performed with iQ<sup>™</sup> SYBR<sup>®</sup> Green Supermix in an iCycler iQ System (Bio-Rad) using the SYBR Green Detection protocol. Total reaction volume was 20 µl and a cycle consists of 95°C for 5 min, 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, for a total of 45 cycles followed by 72°C for 5 min.

#### **Western Blotting**

Detailed method has been described previously [29]. Briefly, cells were washed twice with cold PBS and lysed in RIPA lysis buffer for 30 min on ice. The cells were sheared twice through a 20 gauge needle and centrifuged at 14,000 rpm for 15 min at 4°C. The protein concentration in the supernatant was determined with the Bio-Rad Protein Assay kit. Equal amounts of protein were separated on 10% SDS-PAGE gels, transferred to nitrocellulose membrane with Semi-Dry Transfer Cell (Bio-Rad). The membrane was blocked with TBS containing 5% w/v nonfat dry milk, and hybridized with primary antibody in 2% w/v nonfat dry milk, followed by incubation with secondary antibody and detected with an ECL kit (BioRad).



### Flow Cytometry

To minimize non-specific binding, single cells suspensions were treated with FC block before staining with PE-Cy5 labeled anti-human CD44 antibody for 20 min on ice. After washing with PBS containing 0.5% BSA, the cells were resuspended in the same solution and DAPI was added to a final concentration of 1  $\mu\text{g}/\text{ml}$ . All flow-cytometry studies were performed using either a Becton Dickinson FACS Aria or LSRII flow cytometer. For sorting experiments, the cells were maintained at 4°C during the sort, and an 85  $\mu\text{m}$  nozzle was used. Cells were sorted into RPMI medium. Populations were analyzed post-sort to ensure purity of sorts before progressing with additional experiments. For cells that did not have a clear positive and negative distribution, the top 10% and bottom 10% of cells were sorted and designated as CD44 high and CD44 low.

### Cytospin: Immunofluorescence Analysis

Cytospin preparations of PC cells were fixed in methanol for 10 min at  $-20^{\circ}\text{C}$ , rehydrated in PBS (Sigma-Aldrich Corp., St. Louis, MO; D5773), and blocked in 5% normal goat serum (Rockland Immunochemicals, Inc., Gilbertsville, PA; B304) for 30 min. The slides were incubated with antibodies against CD44 (as above, used at 1:200) and neuron-specific enolase (NSE; Dako North America, Inc.; M0873, 1:50) overnight at 4°C followed by incubation with secondary antibodies (goat anti-rat IgG FITC and Alexa Fluor 546 goat anti-mouse, as described above) for 40 min at room temperature. For cell lines, the slides were mounted with coverslips using Vectashield HardSet Mounting Medium with DAPI. For cells derived from fresh prostatectomy specimens, the slides were stained with Hoechst 33258 (Sigma-Aldrich Corp.; 861405) for 10 min prior to coverslipping. Fluorescence micrographs were obtained with a Leica DM5000 B microscope. Cellular co-expression of CD44 and NSE was quantified in fluorescence micrographs of PC3 and DU145 cytospin preparations. Total cell number was derived by counting nuclei in the DAPI images using the particle analysis feature of NIH ImageJ software (<http://rsb.info.nih.gov/ij/>). Cell masks were generated in ImageJ using a composite of the CD44 and NSE fluorescence signals; the masks were used to derive the mean pixel value of each fluorescence signal within individual cells.

### Statistical Analysis

The analysis included calculation of the Pearson correlations and non-parametric Spearman's correlations between CD44 and NSE levels. Linear regression analysis was also implemented with an assessment of

residuals as a check on the assumptions of normally distributed errors with constant variance. If the assumptions seemed to be violated, log-transformed values were used to produce more normally distributed residuals. Statistical outliers were defined as the standardized residuals values  $>3$  or  $\leq -3$ . Then the models were rerun without the outliers and the results with and without outliers were compared.

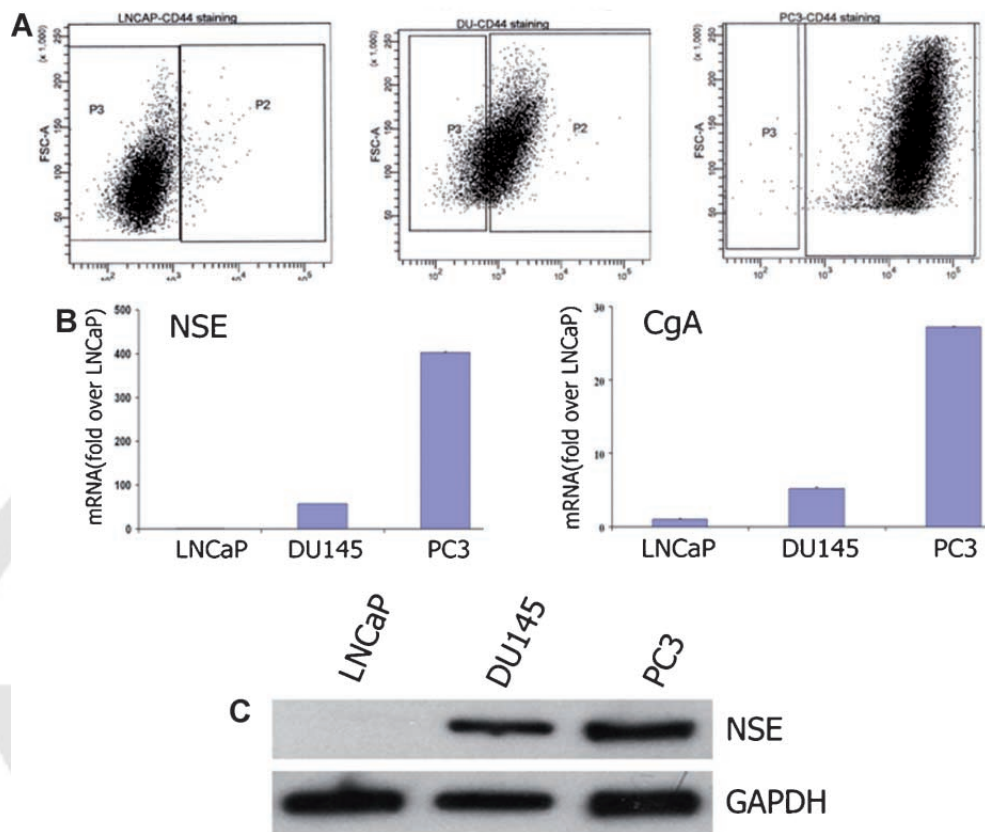
## RESULTS

### Expression of CD44 and NE markers in Human PC Cell Lines

Flow cytometric studies demonstrated that among the three well-characterized PC cell lines (LNCaP, DU145, and PC-3), PC3 cells were nearly 100% positive for CD44 expression, and ~60% of DU145 cells were positive for CD44. LNCaP cells were nearly entirely negative for CD44 (Fig. 1A). These results are consistent with the findings reported by Patrawala et al. [20] We then studied if CD44 expression correlates with NE phenotype in these cell lines. The most commonly used NE markers include chromogranin A and NSE [25]. As shown in Figure 1B,C, the largely CD44 $-$  LNCaP cell line did not express NE markers, while NE marker mRNA was detected, in varying degrees, in the CD44 $+$  DU145 cells and PC3 cells. The observed expression pattern of chromogranin A and NSE mRNAs paralleled that of CD44 expression among the three cell lines (i.e., PC3 had the highest CD44 content and the highest NE marker mRNA concentration).

To further characterize the association of CD44 expression with NE markers, we used fluorescence activated cell sorting (FACS) to sort LNCaP, PC3 and DU145 cells into CD44 high and CD44 low expressing subpopulations. As shown in Figure 2A,B, within each cell line studied, NE marker expression was enriched in the CD44 high population versus unsorted and CD44 low cells. This finding was confirmed with Western blot analysis as depicted in Figure 2C.

We next examined the expression of CD44 and the NE marker NSE in the three cell lines by immunofluorescence after the cells were spun onto glass slides by the cytospin technique. The advantage of this technique is that the expression of multiple proteins can be simultaneously studied in the same cells. As shown in Figure 3A, LNCaP cells were essentially negative for both CD44 and NSE and PC3 cells were nearly all positive for both CD44 and NSE. DU145 cells displayed a wide range of staining, from totally negative to brightly positive for both CD44 and NSE. In general, CD44 negative DU145 cells were negative for NSE while CD44 positive DU145 cells were positive for NSE.



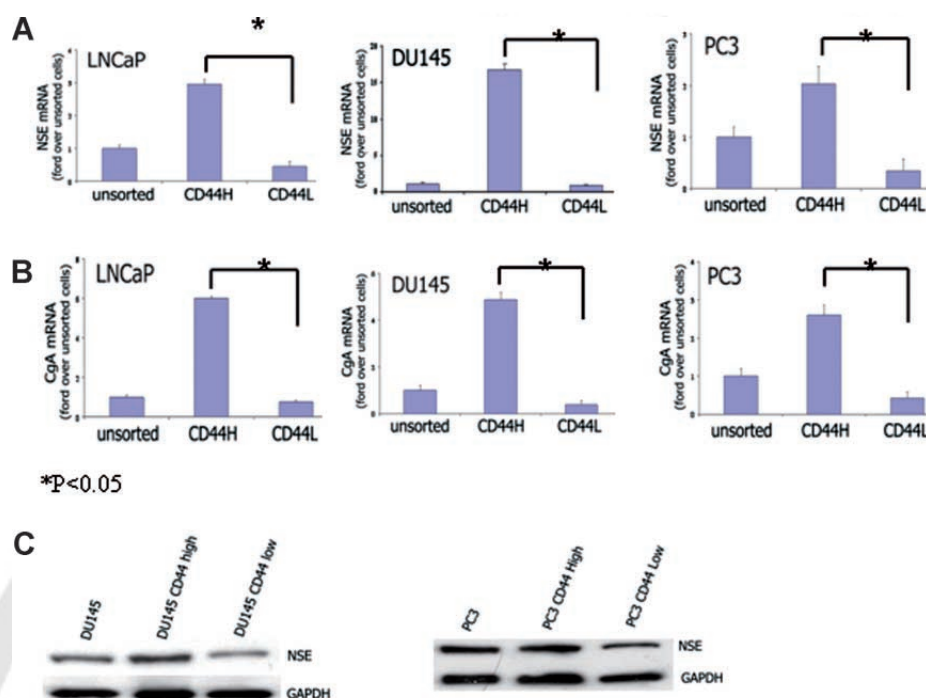
**Fig. 1.** Expression of CD44 and NE cell marker in human prostate cancer cell lines. **A:** Flow cytometry examining CD44 expression in LNCaP, DU145, and PC3 cells. LNCaP cells are mostly negative for CD44. Approximately 60% of DU145 cells are positive for CD44 while PC3 cells are mostly positive for CD44. **B:** RT-PCR analysis of the mRNA levels of NE cell markers in PC cell lines. Expression of NE cell markers neuron specific enolase (NSE) and chromogranin A (CgA) mirrored CD44 expression with LNCaP cells expressing lowest levels while PC3 cells expressed higher levels. **C:** Western blot analysis of the protein levels of NSE in PC cell lines. Similarly, NSE protein was undetectable in LNCaP cells and highest in PC3 cells.

Statistical analysis was performed to study the correlation between CD44 and NSE expression after the image intensity of individual cells was captured, as described in Materials and Methods Section. The correlations between CD44 and NSE were 0.6901 in DU145 cells and 0.6518 in PC3 cells. The correlations based on log-transformed values were similar, 0.6860 and 0.6585 respectively. The non-parametric Spearman correlation was similar for DU145 cells (0.6764), and higher for PC3 cells (0.7516). The linear model for DU145 cells with CD44 as the predictor and NSE as the response had an  $R^2$  of 0.4763, and for PC3 cells the  $R^2$  was 0.4249. Both models were highly significant ( $P < 0.0001$ ). The models identified 3 and 4 outliers for DU145 and PC3 cells respectively. After removing the outliers, the  $R^2$  increased to 0.4944 and 0.5019, respectively. The residual plots showed that the assumption of normal error distribution was satisfactory. Nevertheless, the linear models for log-transformed values were explored and their  $R^2$  values were similar to those without transformation, 0.4705 and 0.4336 respectively. Figure 3B shows

the linear fits based on raw values (without log transformation). These data indicate that on cytospin examination, there is a strong correlation between the expression of CD44 and NSE, suggesting that CD44 expression is associated with NE phenotype in such cells.

#### Expression of CD44 and NE Markers in Primary Fresh Human PC Cells

To further establish the relationship between CD44 expression and NE markers in PC, we obtained fresh PC tissue from seven prostatectomy specimens immediately upon removal of the prostate. Single cell suspensions were obtained and flow-sorted into CD44 high and CD44 low cells. The small number of cells derived from the surgical specimens allowed only quantitative real-time PCR analysis. In every case, the levels of NE markers were much higher in the CD44 high cells than those in the CD44 low cells and the difference was statistically different in each case (Fig. 4A,B).



**Fig. 2.** Expression of NE markers in CD44 high and CD44 low fractions of human prostate cancer cell lines. RT-PCR for mRNA levels of NSE (**A**) and CgA (**B**) revealed enrichment for NE marker expression in CD44 high (CD44H) populations versus CD44 low (CD44L) and unsorted populations. This finding was confirmed at the protein level by Western blot for NSE (**C**). The low level of CD44 expression within LNCaP cells precluded accurate sorting and adequate protein extraction from CD44 + LNCaP cells for protein analysis.

The single cell suspensions from fresh PC tissue were also spun onto slides by cytopsin method and double-stained by immunofluorescence for the expression of CD44 and chromogranin A. As predicted, very few cells were NE cells. Similarly, in these single cell suspensions, CD44 expression was limited to NE tumor cells (Fig. 4C).

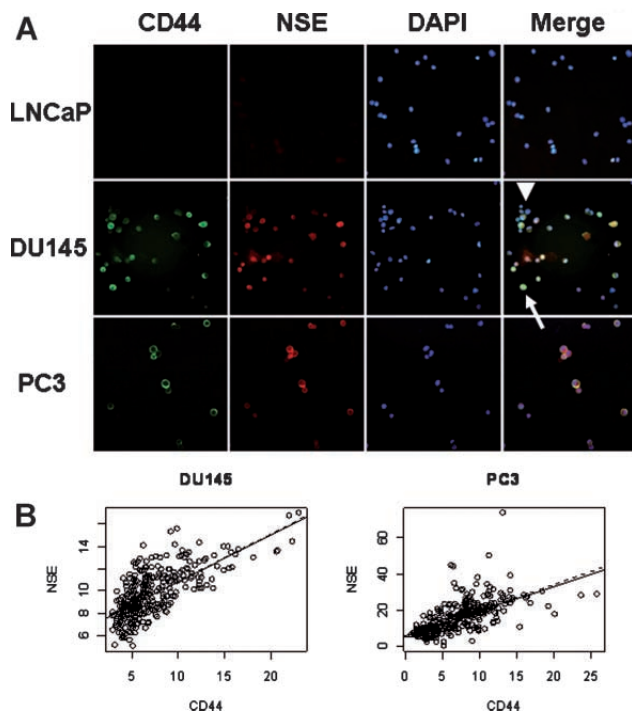
#### Expression of CD44 in Benign and Malignant Prostate Tissue

We then performed immunohistochemistry to study the expression of CD44 in archival, formalin-fixed and paraffin-embedded sections of human PC. Positive staining was defined as strong membrane staining, consistent with CD44 being a cell surface protein. In benign prostate tissue, all basal cells expressed CD44, consistent with previous reports [30–32] (Fig. 5A). Lymphocytes and nerves were also positive for CD44 (Fig. 5B,C). PC is characterized by the absence of basal cells and the proliferation of luminal type malignant epithelial cells. Although the majority of cancer cells were negative for CD44, there were scattered individual cells or small nests of cells that displayed CD44 expression with a distinct membranous staining pattern. The distribution of the CD44 + cells was reminiscent of NE tumor cells of PC (Fig. 5D).

#### Co-Expression of CD44 and Chromogranin A in Human PC Tissue

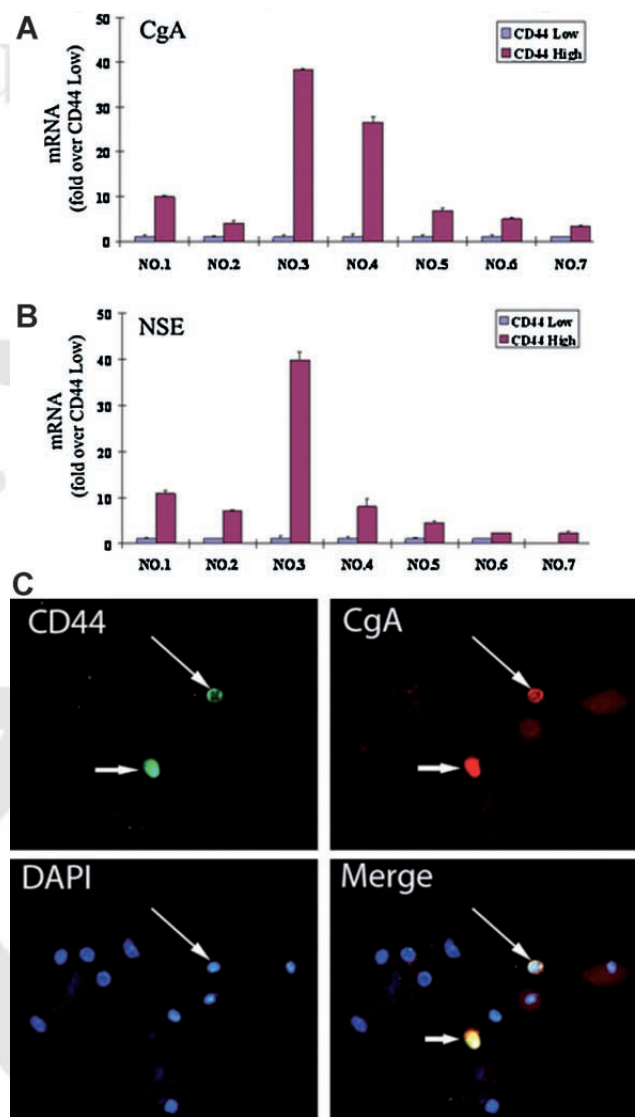
We next performed experiments to confirm that CD44 + cells in PC tissues are in fact NE cells. We prepared adjacent sections of human PC tissue (5  $\mu$ m apart) which contained virtually identical tumor cells. The first section was stained with an anti-CD44 antibody and the second section stained with an anti-chromogranin A antibody to highlight NE cells. Chromogranin A positive NE cells displayed cytoplasmic staining and were scattered among the more abundant cancerous epithelial cells. In the adjacent section, CD44 + cells demonstrated a membrane staining pattern and similarly appeared as single cells and small nests of cells surrounded by more abundant CD44 – cells. When the same microscopic fields from the two adjacent sections were compared, cells that were positive for CD44 were also noted to be positive for chromogranin A and vice versa (for illustration, an area with abundant NE cells are shown in Fig. 6A).

In order to definitively prove the relationship of CD44 expression with NE cells in PC, we employed an immunofluorescence method so that multiple antibodies could be used to stain the same tumor cells. Our pilot studies indicated that NE cells within tumors were all positive for CD44 but CD44 positive cells were



**Fig. 3.** Co-expression of NSE and CD44 in human prostate cancer cell lines. **A:** Immunofluorescence studies on cytospin samples with antibodies against CD44, NSE (with DAPI staining nuclei) show co-expression of CD44 and NSE in the same cells. LNCaP cells are double negative for the two markers and PC3 cells are double positive. The majority of DU145 cells are double positive (arrow) but a minority are double negative (arrowhead) (magnification 400 $\times$ ). **B:** Linear fits of CD44 and NSE for DU145 and PC3 cells. The linear model with CD44 as the predictor and NSE as the response for DU145 cells yields an  $R^2$  of 0.4763 and for PC3 cells 0.4249. Both models are highly significant ( $P < 0.0001$ ). The dash line is the fit with outliers and the solid line without outliers. These data indicate that on cytospin examination, CD44 and NSE expressions were closely associated with each other in individual cells.

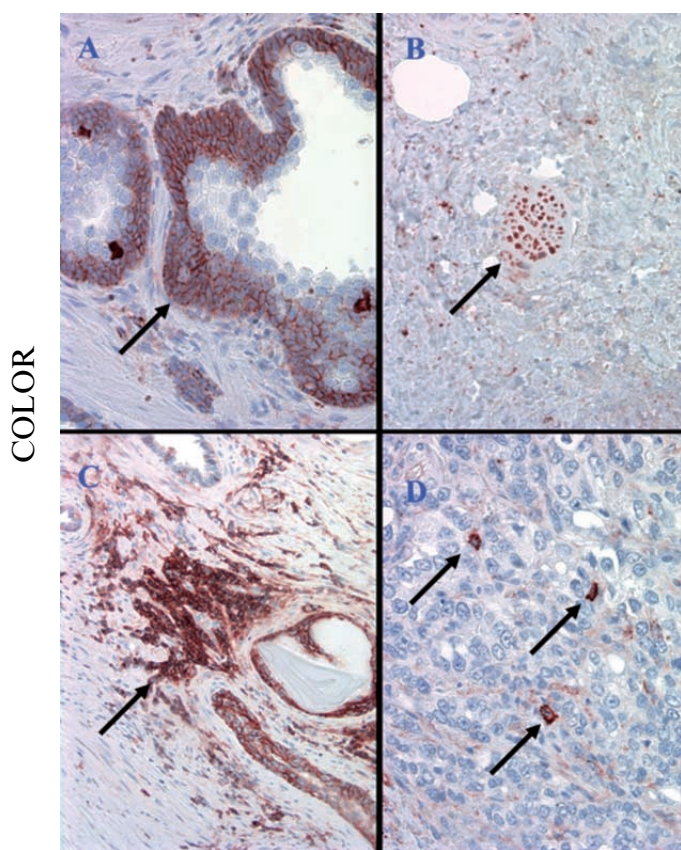
composed of NE tumor cells and lymphocytes that commonly infiltrate PC. Therefore, we co-stained a section of a tissue microarray that contained 200 cores of PC tissue from 73 different radical prostatectomy cases for the expression of CD44, chromogranin A and CD45 (a marker of leukocytes including lymphocytes). The areas of cancer in each core were marked and the number of nuclei (stained by DAPI, including cancer cells + lymphocytes) in cancerous areas of each core counted manually, which ranged from 40 to 1,755 per core with a total of 61,070 cells surveyed in aggregate. Among them, 147 cells were positive for chromogranin A (NE cells) comprising 0.2% of all nuclei. Of these, 132 (89.8%) were CD44+. Lymphocytes (CD45+) comprised 0.8% (516 cells) of all nuclei (Table I). Approximately 10% (15 cells) of NE cells were negative for both CD44 and CD45. Among the 648 CD44+ cells counted, 132 (20.4%) were positive for chromogranin A,



**Fig. 4.** Association of CD44 expression with NE cells in fresh primary human prostate cancer cells. **A:** Quantitative RT-PCR analysis performed on sorted single cell suspensions obtained from seven cases of fresh radical prostatectomy specimens revealed that NE markers CgA and NSE expression was significantly higher in the CD44 high versus the CD44 low population. **B:** Single cell suspension obtained from a case of fresh radical prostatectomy specimen was co-stained by immunofluorescence for the expression of CD44 and CgA (nuclei stained by Hoechst 33258). A single NE cell is the only CD44+ cell (long arrow). The other bright spot (short arrow) in the field is a contaminant as it is not associated with a nucleus (magnification 400 $\times$ ).

516 (79.6%) were positive for CD45 and 2 (0.3%) were positive for both chromogranin A and CD45 (faint) (Table I). Of the 61,070 cells reviewed, 2 were faintly triple positive for CD44, CD45 and CgA. These two cells were not included in the above analysis. Therefore, with few exceptions, NE tumor cells were CD44+ cells; and CD44+ cells, minus a population of lymphocytes, were





**Fig. 5.** Immunohistochemical study of the expression of CD44 in benign prostate and prostate cancer. In benign prostate, expression of CD44 is seen in (A) basal cells (arrow); (B) nerve (arrow); (C) lymphocytes (arrow). In prostate cancer (D), expression of CD44 is seen in scattered tumor cells, reminiscent of the distribution of neuroendocrine tumor cells (magnification 400 $\times$ ).

all NE tumor cells (Fig. 6B). A representative area of PC with lymphocytes (CD45 + /CD44 + /CgA -) and an NE cell (CgA + CD44 + /CD45 -) is shown in Figure 6C.

## DISCUSSION

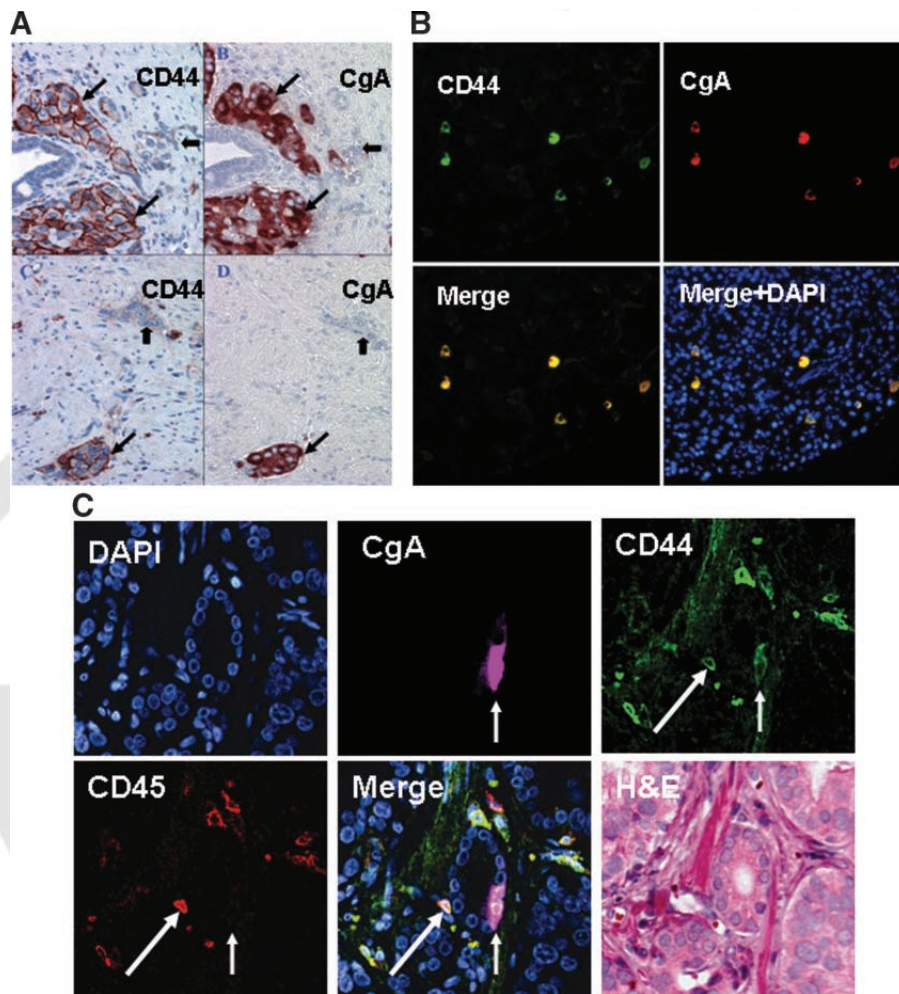
The mechanisms by which PC cells proliferate in an androgen-deprived environment remain unclear. Current hypotheses focus largely on altered AR signaling in tumor cells, including amplification of the AR gene, increased AR protein stability, AR hypersensitivity to low levels of androgen, AR mutation and activation of mutant AR by non-traditional ligands (reviewed by Scher and Sawyers [33]). An alternate theory that has gained significant attention recently involves CSCs. The hierarchical CSC model predicts that the putative PC stem cell, unlike the bulk tumor cells, is AR negative and androgen-independent. As a result, PC stem cells may be resistant to hormone ablation and responsible for tumor recurrence. Although many different

markers have been reported to identify CSCs in PC [10–16,34–36], the comprehensive study by Patrawala et al. [20] as well as those by others, have provided convincing evidence that the CD44 + subpopulation of cells may demarcate the PC stem/progenitor cells.

PCs are composed mostly of secretory type epithelial tumor cells with a small population of morphologically and functionally distinct NE cells. NE cells are increased in high grade and high stage tumors, particularly in hormonally treated and hormone-refractory tumors [25]. The levels of circulating chromogranin A, a product of the NE cells, are increased in men with PC in comparison to patients with benign conditions. Furthermore, serum chromogranin A levels correlate with the stage of disease and is an independent prognostic factor in men with hormone-refractory disease [25]. An important feature of NE cells is that they do not express AR [22–24]. Thus, they may be resistant to androgen ablation and contribute to tumor recurrence after hormonal therapy. Animal studies using xenograft and genetic PC models support this view. Huss et al. reported that in the CWR22 human PC xenograft model, castration induces tumor regression followed by recurrence (androgen-independent tumor outgrowth). Notably, these investigators observed an increase in the number and proliferative activity of tumor NE cells after castration, suggesting that NE cells may promote tumor survival and resurgence [37]. Genetic animal models of PC also contain NE cells varying from very low in Pten -/- tumors [38] to high in tumors of TRAMP [39] and Rb-p53-mice [40]. Similarly, recurrent tumors in Pten -/- tumors after castration have been shown to be composed of significantly more NE cells than pre-castrate primary tumors [38].

In the current study, we have for the first time demonstrated unequivocally that NE cells are the only CD44 + tumor cells (i.e., non-lymphocyte/CD45 -) in human PC tissue. In addition, we have ascertained an association of CD44 expression with cells expressing NE markers in three well-established human PC cell lines. Patrawala et al. [20] have shown that the AR-DU145 and PC3 cell lines, but not the AR + LNCaP cell line, express CD44. Leiblich et al. [41] found that NE markers are expressed in DU145 and PC3 cells, but not in LNCaP cells. Our results are consistent with these reports and indicate that in human PC cell lines, expression of the stem/progenitor cell marker CD44 is associated with cells with NE features. Furthermore, we confirmed the expression of NE markers from CD44 + cells in single cell suspensions obtained from fresh human surgical samples and human PC tissues at both the RNA and protein levels.

Using immunohistochemical and immunofluorescence studies of archival PC tissue in a tissue microarray, we showed that, excluding infiltrating



**Fig. 6.** Expression of CD44 is limited to NE tumor cells in human prostate cancer tissues. **A:** Immunohistochemical study of adjacent sections of a PC TMA for the expression of CD44 and CgA to show that NE tumor cells are CD44+ (long arrow) while non-NE tumor cells are CD44- (short arrow). **B:** A PC TMA slide was co-stained for the expression of CD44, CD45 and CgA by immunofluorescence study. In this

lymphocytes (CD44 and CD45 double positive cells), expression of CD44, a putative CSC marker, is confined to NE tumor cells, an important observation that strengthens the hypothesis that NE cells within prostate tumors, being AR/PSA negative and normally quiescent [24,42], are possibly the therapy resistant cells responsible for tumor recurrence. These results are consistent with our recent finding that small cell

carcinoma of the prostate, a tumor that is composed of pure malignant NE cells, consistently expresses CD44 [43].

A recent publication shows that p53 inhibits expression of the CD44 to allow an untransformed cell to respond to stress-induced, p53-dependent cytostatic and apoptotic signals. In the absence of p53 function, the resulting CD44 expression is essential for the

**TABLE I.** Expression of CD44, CgA, and CD45 in Cancer Areas of Human PC TMA (Total Nucleated Cells = 61,070)

	CgA + (NE cells) (n = 147)	CD44 + (NE cells + lymphocytes) (n = 648)	CD45 + (lymphocytes) (n = 516)
CgA- (n = 60,923)	—	516 (79.6%)	516 (100%)
CD44- (n = 60,407)	15 (10.2%)	—	0 (0%)
CD45- (n = 60,554)	147 (100%)	132 (20.4%)	—



growth and tumor-initiating ability of highly tumorigenic mammary epithelial cells [44]. Significant expression of CD44 in NE tumor cells of PC suggests that these cells may be highly tumorigenic, as has been proposed for CSCs, challenging the concept that NE tumor cells are terminally differentiated, post-mitotic and play no role in cancer progression. This hypothesis is also consistent with the observation by Patrawala et al. [20] that the CD44+, AR- PC cells can give rise to CD44-, AR+ cells.

The reverse analysis showed that approximately 90% of the NE cells express CD44 while the remaining 10% were CD44-. Although this suggests the possibility of heterogeneity within the NE population, we cannot rule out false negative CD44 staining in some NE cells due to a sample bias based upon technical issues. For example, tumor cells in tissue section may not have been uniformly sectioned and hence focal membrane staining for CD44 may be missed in rare cells.

The origin of NE cells in the prostate remains controversial. NE cells are present in benign prostate as well as all stages of prostatic carcinogenesis, from PIN [45] to invasive carcinoma to metastatic PC [46,47]. It has been proposed that they may be derived from the same stem cell or pluripotent cell that gives rise to luminal secretory cells [48,49]. A population of proliferating/transit amplifying intermediate cells has been identified and postulated to be a common precursor for NE cells and other epithelial cells of the benign prostate [50,51]. The same has been assumed for the NE cells in PC which are considered to share the same stem/precursor cells with the secretory type cancer cells; although no definitive experimental evidence has been reported. Alternatively, some investigators favor the trans-differentiation model of NE cell origin, which suggests that the tumor NE cells are derived from the non-NE secretory-type tumor cells. For example, in vitro assays, LNCaP cells, an androgen-dependent cell line, can be induced to show NE-like phenotype by androgen deprivation [52] or agents that increase intracellular levels of cAMP [53]. Our results, in combination with recent publications, would suggest an entirely different view, that is, at least in cancer, NE cells may themselves represent the stem/progenitor cells for the bulk differentiated, secretory type cancer cells. This may have profound implications on the treatment of PC as it suggests that only therapies that target NE cells, in combination with hormonal therapy that target the bulk tumor cells, would have the potential of curing men with lethal PC.

The CSC concept may have different meanings in different contexts. As summarized by Jordan et al. [5] CSCs can (i) be the source of all tumor cells in a primary tumor, (ii) comprise the small reservoir of therapy-resistant cells that are responsible for tumor recurrence

after therapy-induced remission, and/or (iii) give rise to metastatic tumors. Because of the difficulty associated with purifying NE cells from fresh human PC tissue, functional studies on NE cells have not been reported. However, current evidence suggests that they may represent the hormonal therapy-resistant cells that are responsible for tumor recurrence; thus fulfilling a functional definition of a CSC. Based upon the present study, further functional and mechanistic studies are warranted to establish the role of NE cells as the putative PC stem cell.

## CONCLUSION

We have provided strong evidence that CD44, a marker that has been shown to be associated with increased tumorigenic potential in PC cell line and xenograft tumors, is expressed selectively in NE cells of human PC. This finding, in combination with the fact that such tumor cells do not express AR and are likely androgen-independent, further suggest their potential roles in tumor recurrence after hormonal therapy.

## ACKNOWLEDGMENTS

G.S. Palapattu was supported by Department of Defense Prostate Cancer Research Program (PC073121) and AUA Foundation/Astellas Rising Stars in Urology Award. J. Huang was supported by grants from American Cancer Society (RSG-07-092-01-TBE) and Department of Defense Prostate Cancer Research Program (PC061456).

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